

GENERATION AND CHARACTERIZATION OF BIOACTIVE PEPTIDES FROM
FLAXSEED (*Linum usitatissimum* L.) PROTEINS

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ABSTRACT

The potential of flaxseed (*Linum usitatissimum* L.) protein to release bioactive peptides upon enzymatic hydrolysis was evaluated. Flaxseed protein released angiotensin I-converting enzyme inhibitory (ACEI) peptides during *in vitro* simulated gastrointestinal (GI) digestion in a static (no removal of digested products) and a dynamic model (removal of <1 kDa molecules in the intestinal phase). The ACEI activity of the gastric plus intestinal digest (absorbed fraction-IC₅₀: 0.04 mg N/mL; retained fraction-IC₅₀: 0.05 mg N/mL; degree of hydrolysis, DH: 46.78 %) of the dynamic model was significantly higher ($P<0.05$) than that of the static model (IC₅₀: 0.39 mg N/mL; DH: 43.95 %). Polypeptides of 48, 41, 29 and 20 kDa could be releasing these ACEI peptides. Six peptides in the highest ACEI fraction (0.5-1 kDa) of the absorbable gastric plus intestinal digest were identified via *de novo* sequencing. Only digests of the static model exhibited hydroxyl radical (OH[•]) scavenging activity (IC₅₀: 0.40 mg N/mL), suggesting the inappropriateness of such models in this type of research. Presence of mucilage and oil interfered with the *in vitro* digestibility of flaxseed protein, which could limit the release of ACEI peptides during GI digestion. The protein digestibility of milled whole flaxseed (12.61 %) was significantly improved ($P<0.05$) with the removal of mucilage (51.00 %) and oil together with mucilage (66.79 %). The digestibility of isolated flaxseed protein was 68.00 %.

Flaxseed protein, hydrolyzed (DH: 11.94-70.62 %) with Flavourzyme[®] in a central composite rotatable design, possessed bioactivities with identified optimum enzyme/substrate and time of hydrolysis combinations, including ACEI activity (71.59-88.29 %, 83.7 LAPU/g protein, 19.9 h), scavenging of OH[•] (12.48-22.08 %, 30.2 LAPU/ g protein, 1.5 h) and superoxide radical (O₂^{•-}) (26.33-39.41 %, 4.9 LAPU/ g protein, 16.3 h) and inhibiting linoleic acid oxidation (0.71-94.33 %, 1.6 LAPU/ g protein, 12.6 h). The degradation pattern of polypeptides during enzymatic hydrolysis indicated that 48 and 13 kDa molecules could be releasing these bioactive peptides. *De novo* sequencing identified two ACEI and five OH[•] scavenging peptides in the hydrolysate fractions (0.5-1.05 kDa) with the highest bioactivities. The findings suggest the importance of flaxseed protein as a source of cardioprotective bioactive peptides.

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LIST OF ABBREVIATIONS

ABTS^{•+}: 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

ACE: Angiotensin I-converting enzyme

ACEI: Angiotensin I-converting enzyme inhibitory

ADP: Adenosine diphosphate

ALA: ∞ Linolenic acid

ANOVA: Analysis of variance

AOAC: Association of Official Analytical Chemists

BHA: Butylated hydroxyanisole

BHT: Butylated hydroxytoluene

CaM: Calmodulin

CCRD: Central composite rotatable design

CGP: Caseinoglycopeptide

CHD: Coronary heart disease

CVD: Cardiovascular diseases

DBP: Diastolic blood pressure

dGIF: Dynamic model- Gastric+intestinal fraction

DH: Degree of hydrolysis

DNA: Deoxyribonucleic acid

DPPH[•]: 2, 2-diphenyl-1-picrylhydrazyl radical

DR: Deoxyribose

E/S: Enzyme to substrate ratio

E/T %: The percentage ratio between essential and total amino acids

ESI: Electrospray ionization

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration
FDR: Food and Drug Regulations
FPH: Flaxseed protein hydrolysate
FPLC: Fast protein liquid chromatography
GF: Gastric fraction
GI: gastrointestinal
GRAS: Generally recognized as safe
GSH: Glutathione
HMW: High molecular weight
HPLC: High performance liquid chromatography
HPX: Hypoxanthine
HSD: Hydroxyl-steroid dehydrogenase
IDF: Insoluble dietary fibre
IEP: Isoelectric pH
LAPU: Leucine aminopeptidase units
LDL: Low density lipoprotein
LMW: Low molecular weight
ME: mercaptoethanol
MS: Mass spectrometer
MWCO: Molecular weight cut off
NBT: Nitrotetrazolium blue
nNOS: Nitric oxide synthase
NR: Not reported/Non-reducing
O.D.: Optical density
 $O_2^{\bullet -}$: Superoxide radical

OH[•]: Hydroxyl radical

OPA: O-phthalaldehyde

PCL: Peptide chain length

QSAR: Quantitative structure activity relationship

RAS: Renin angiotensin system

ROS: Reactive oxygen species

SBP: Systolic blood pressure

SC: Sodium Cholate

SCDC: Sodium chenodeoxycholate

SDC: Sodium deoxycholate

SDF: Soluble dietary fibre

SDS- PAGE: Sodium dodecyl sulphate- polyacrylamide gel electrophoresis

SGC: Sodium glycocholate

SGF: Simulated gastric fluid

sGIF: Static model-Gastric+intestinal fraction

SIF: Simulated intestinal fluid

SPH: Soy protein peptic hydrolysate

SPHP: Soy protein peptic hydrolysate with phospholipids

SSP: Seed storage protein

STC: Sodium taurocholate

TDF: Total dietary fibre

UPLC: Ultra performance liquid chromatography

USP: United States Pharmacopeia

XOD: Xanthine oxidase

1. INTRODUCTION

Flaxseed (*Linum usitatissimum* L.) has received attention among consumers and health care professionals alike due to reported health benefits associated with its consumption. Some of these health benefits include reduction of atherogenic risk, anticancer, antiviral, bactericidal, anti-inflammatory and laxative effects (Collins *et al.*, 2003).

Flaxseed is rich in oil, protein, soluble fibre (mucilage/gum) and lignans. The major product obtained from flaxseed is oil, which is rich in the omega 3 (ω -3) fatty acid, α -linolenic acid (ALA). Currently, ground or whole flaxseed and defatted meal are marketed for food applications as a convenient source of dietary fibre, lignans, and soluble fibre. Whole flaxseed has 23 % protein (dry matter basis) on average (Flax Council of Canada, 2010). The defatted meal which is the byproduct from oil extraction contains 35-40 % protein (Sammour, 1999). However, the proteins of flaxseed meal have not yet been widely exploited for their value in human nutrition.

Whole flaxseed, its meal as well as flaxseed constituents have demonstrated protection against cardiovascular disease (CVD) (Jenkins *et al.*, 1999). Several studies have been conducted to identify the constituents in flaxseed, which contributed to heart health related benefits. Most of these studies focused on and relating to the ω -3 fatty acid, ALA in flaxseed oil, lignan and mucilage (Bloedon & Szapary, 2004; Prasad, 2005). However, findings of these studies are variable and inconsistent and the specific components of flaxseed, which contribute to these health benefits, has not been completely elucidated. Recent studies (Hallund *et al.*, 2006; Sano *et al.*, 2003) have challenged the hypothesis that ALA and lignans are the components responsible for the health benefits of flaxseed. This has raised interest in identifying the exact component.

in flaxseed leading to health benefits (Jenkins *et al.*, 1999; Sano *et al.*, 2003). The exerted antiatherogenic effect by defatted flaxseed meal but not by isolated lignans suggests that a non-lipid and non-lignan component in flaxseed meal could be the contributory factor for protection against CVD (Jenkins *et al.*, 1999; Sano *et al.*, 2003). Within the context of dietary health beneficial components of flaxseed, it is necessary to investigate the contribution of the protein fraction, especially the bioactivities of the digested protein products.

Bioactive peptides are the specific protein derived fragments that have positive impact on body health (Kitts & Weiller, 2003). These peptides are inactive within the sequence of parent proteins but become active when released during gastrointestinal (GI) digestion or food processing. Upon release they exert various physiological functions based on the inherent amino acid composition and sequence. It is well accepted that 30-50 % of dietary nitrogen is absorbed into the body as small peptides, which can also exert different bioactivities (Roberts *et al.*, 1999). At present, several enzymatically hydrolyzed food proteins have been studied for their ability to release bioactive peptides with cardioprotective activities. Some of these activities include angiotensin I-converting enzyme inhibitory (ACEI) activity (antihypertensive), antioxidant, bile acid binding, and antithrombotic activities.

There are several published studies reporting the presence of bioactivities in enzymatically hydrolyzed flaxseed protein. Some examples are *in vitro* ACEI, renin inhibitory (Udenigwe *et al.*, 2009) and antioxidative activity (Udenigwe *et al.*, 2009) of flaxseed protein hydrolyzed with ficin, pancreatin, papain, pepsin and Thermolysin. Wu *et al.* (2004) reported in a patent that proteolytic digestion of defatted flaxseed meal generates a hydrolysate with ACEI activity. Also Omoni and Aluko (2006) reported *in vitro* calmodulin binding activity and calmodulin dependent neuronal nitric oxide synthase inhibitory activity of an Alcalase-hydrolysate of flaxseed protein. However, there is no published information available on the ability of flaxseed protein to generate cardioprotective bioactive peptides during GI digestion.

Flaxseed is consumed as whole flaxseeds, ground (milled) flaxseeds or defatted flaxseed meal. In whole flaxseed, the protein is present with other seed components such as mucilage and oil, whereas in the meal the oil is expressed out. Hence, the form in which flaxseed is consumed might affect the bioactivity of flaxseed protein derived peptides. Further, the use of food grade microbial enzymes that can extensively hydrolyze proteins may generate beneficial bioactive peptides. This information has the potential to add more value to flaxseed meal, which has not been commercially exploited as a protein source.

In this study, three hypotheses were tested in relation to the generation of health beneficial bioactivities by flaxseed proteins. These hypotheses and objectives were as follows.

Hypothesis 1: Seed proteins of flaxseed release bioactive peptides upon GI digestion that are resistant to further hydrolysis by digestive enzymes and have ACEI and antioxidant activities.

Objectives:

- To determine whether flaxseed protein releases peptides with ACEI and antioxidant [hydroxyl radical (OH[•]) scavenging] activities during the simulated GI digestion *in vitro*.
- To identify the molecular mass and amino acid sequence of the peptides in the fractions with the highest levels of these bioactivities and released from *in vitro* digestion of flaxseed protein.

Hypothesis 2: Digestibility of flaxseed proteins during simulated GI digestion is reduced by the presence of other constituents in the seed matrix, *i.e.* reduce the release of bioactive peptides.

Objectives:

- To investigate the digestibility of different forms of flaxseed protein sources (milled whole flaxseed, milled and demucilaged flaxseed, milled, demucilaged and defatted flaxseed and isolated protein).

- To determine the impact of presence of other seed components (mucilage and oil) on the digestibility and degradation of flaxseed proteins under simulated GI digestion.

Hypothesis 3: Exogenous hydrolysis of flaxseed proteins with commercial fungal proteases can generate bioactive peptides with multiple cardioprotective activities.

Objectives:

- To determine whether peptides released during hydrolysis of flaxseed proteins by Flavourzyme[®] have bioactivities such as ACEI, anti-oxidative, anti-thrombotic activities and bile acid binding ability and to identify their precursor proteins.
- To identify the optimum reaction conditions that produce peptides with maximum activities for each of the bioactivities screened.
- To identify molecular masses and amino acid sequences of peptides with bioactivities.

Studies were designed, conducted and results were analyzed to test these hypotheses.

2. LITERATURE REVIEW

2.1 Flax as a crop

Flax (*Linum usitatissimum*) is a blue flowered annual oilseed crop, which belongs to the family, *Linaceae*. The flax plant is native to Western Asia and the Mediterranean (Coskuner & Karababa, 2007) and grown as a crop in many cool climate regions of the world except the tropics and the arctic (Madhusudhan, 2009). In Canada, flax is grown mainly in the cool northern climate of the Western prairies. During ancient times flax plant was primarily used to obtain flax fibre. Around 6000 BC flax was used in Eastern Turkey to make cloth known as linen (Judd, 1995). These linen cloths were used to wrap the mummies in the early Egyptian tombs and flax spinning and weaving had been household industries in antiquity (Oplinger *et al.*, 1989). However, with the invention of cotton, fibre flax production declined and the major use of flax was switched from fibre to oil extracted from the seeds. Traditionally, the flax oil was used in the manufacture of paints, varnishes and linoleum, due to its drying and hardening properties upon exposure to air and sun light (Coskuner & Karababa, 2007). In addition consumption of seeds of flax in various forms as a food ingredient and for its medicinal properties dates from 5000 BC since the start of its cultivation (Oomah & Mazza, 1998).

Today, Canada is the world leader in the production and exports of flax (Flax Council of Canada, 2010) contributing about 50 % of the world flax production and representing about 80 % of the global flax trade (Agriculture and Agri-Food Canada, 2010). Canadian flax is exported mainly to Europe, the United States, Japan, and South Korea (Flax Council of Canada, 2010). The major areas in Canada that cultivate flax include Alberta, Manitoba, New Brunswick, Nova Scotia, Prince Edward Island and

Saskatchewan (Canadian Grain Commission, 2010). Saskatchewan leads Canadian commercial flax cultivation by contributing 70 % of the total production and representing 25 % of world production (Government of Saskatchewan, 2010).

2.2 Flaxseed; Structure and composition

The seeds of flax are known as flaxseeds (also known as linseed) and are small, flat, oval shaped and pointed at one end. The seed has a smooth glossy surface and its colour ranges from medium, reddish brown to a light yellow. The seed dimension is approximately 3.0-6.4 mm in length, 1.8-3.4 mm in width and 0.5-1.6 mm in thickness (Freeman, 1995). Oily flaxseed is larger in size than fibre flaxseed (Green & Marshall, 1981). The seed contains a seed coat (testa, true hull or spermoderm), an embryo or the germ, a thin endosperm and two large, flattened cotyledons. The cotyledons form the bulk of the seed (57 %) while the seed coat and the endosperm account for 38 % and the embryo comprises 5 % of the total seed weight (Wanasundara & Shahidi, 1998). The seed coat of flax has five distinct layers. The epidermal layer or the mucilage layer and the testa, which consists of pigmented cells that give the flaxseeds its color, are the two important layers of the seed coat.

Flaxseed is rich in oil, protein and dietary fibre. The seed has 30-40 % oil, 20-25 % protein, 20-28 % dietary fibre, 4-8 % moisture, and 3-4 % ash (Bhatty, 1997; Carter, 1993; Cunnane *et al.*, 1993; Oomah & Mazza, 1998). Cotyledons are the major storage site for flaxseed oil (contain 75 % of the seed oil). The seed coat and the endosperm contain 22 % of the seed oil while the embryo contains only 3 % of the oil (Wanasundara & Shahidi, 1998). Flaxseed is naturally low in saturated lipids and nearly 73 % of its constituent fatty acids are polyunsaturated. It has a lesser amount of linoleic acid, an ω -6 fatty acid. Flaxseed oil is the richest known source of α linolenic acid (ALA), which is an ω -3 fatty acid, (52 % of total fatty acids). The ω -3/ ω -6 ratio of flaxseed oil is 1:0.3. It has superior ω -3 fatty acid content when compared with the fatty acid profile of algal, corn, fish or soybean oils (Madhusudhan, 2009). Flaxseed is also a rich source of protein, which has amino acid contents similar to that of soybean (Madhusudhan, 2009). The protein content of defatted flaxseed meal ranges from 35-40

% (Sammour, 1999). Similar to other oilseeds, albumins and globulins are the major storage proteins of flaxseed, contributing about 58-66 % of the total seed protein (Chung *et al.*, 2005). Flaxseed proteins are mainly stored in the cotyledons (Wanasundara & Shahidi, 1998)

The dietary fibre in flaxseed is comprised of both insoluble and soluble forms. Flaxseeds contain about 400 g/kg total dietary fibre (TDF) rich in pentosans and the hull fraction contains 2-7 % soluble dietary fibre (SDF) known as mucilage/gum (Bhatta & Cherdkiatgumchai, 1990). The mucilage, which is a mixture of acidic and neutral polysaccharides consisting primarily of D-galacturonic acid, D-xylose, L-rhamnose, D-galactose, and L-arabinose (Mazza & Oomah, 1995), occurs mainly in the epidermal layer of the seed coat (Dev & Quensell, 1988).

Another important group of constituents in flaxseed are the phenolic phytoestrogens called lignans. The major flaxseed lignan is known as secoisolariciresinol diglucoside (SDG) which is present at levels 75-800 times higher than any other crop and vegetable known to-date (Thompson *et al.*, 1997; Westcott & Muir, 1996). The defatted flaxseed flour was shown to contain 1–3 % of SDG (Westcott & Muir, 1996).

2.3 Food and feed uses of flaxseed

Flaxseed products are found in forms of whole seed, ground (milled), partially defatted meal and oil (Bassett *et al.*, 2009). When added to products such as waffles, pancakes, breakfast cereals, drinks, biscuits, crackers, soups, and bagels, etc. ground or whole flaxseed gives a nutty flavor (Berglund, 2002). The ground flaxseed is presently sold in the market under vacuum packaging. Use of ground flaxseed is beneficial over the whole flaxseed as the whole seed can pass through the body undigested (Flax Council of Canada, 2010). In Southern India, flaxseed is consumed as flaxseed chutney (a pickle of Indian origin), which could be preserved for months (Madhusudhan, 2009).

Flax oil is obtained by mechanical pressing of seeds. Traditionally the oil extracted from flaxseed (known as linseed oil) was used for industrial purposes such as drying

agents in paints and varnish (Judd, 1995). With the discovery of health benefits of ALA, oil extracted from cold pressing of flaxseed is now available in super markets and health food stores as bottled oil and sealed gel capsules. The oil can be used in fresh salads whereas the capsules are sold as dietary supplements (Flax Council of Canada, 2010). Use of flax oil for frying is not recommended. Once extracted from the seeds, the polyunsaturated fatty acids (PUFAs) in the flax oil may undergo thermal oxidation when exposed to high temperatures of cooking resulting in off flavours and odours in the finished product (Wanasundara & Shahidi, 1998).

The residue that remains after crushing flaxseed to obtain oil is the flaxseed meal. Most of the flaxseed protein is concentrated in the meal. It also contains fibre and lignans and is poor in ALA content (Bassett *et al.*, 2009). Historically, the meal was used as a livestock feed and fertilizer. Today, the packaged meal is available in health food stores or via internet agents as a food ingredient that can be added to cereals, salads, blended drinks etc. (NexTag, 2010). Although the meal is rich in proteins it is marketed by highlighting its value as a good source of dietary fibre, lignans and mucilage.

Milled whole flaxseed and flaxseed meal are also popular ingredients in animal feed rations. Due to its unique ALA content, farmers and animal breeders include flaxseed in the diet formulations for cattle, equine and hogs to improve animal health. Also feeding hens with poultry feed containing flaxseed is popular to produce eggs rich in ω -3 fats that are appealing to consumers (Flax Council of Canada, 2010).

In January 2009, whole and milled flaxseed was given GRAS (generally recognized as safe) status by the United States Food and Drug Administration (FDA). To be accepted by the food industry in the U.S.A., a new food ingredient has to obtain GRAS status or be free of harmful toxins or unsafe side effects. Gaining this status will further expand the use of flaxseed by food manufacturers and human food applications worldwide (Flax Council of Canada, 2009).

2.4 Flaxseed as a functional food

Foods are now being researched intensively for added physiological benefits that may reduce chronic disease risk or otherwise optimize health. Such research efforts have led to global interest in the food category known as functional foods. There is no universally accepted definition for functional foods. Health Canada defines a functional food as a food similar in appearance to a conventional food, which is consumed as a part of the usual diet and has demonstrated physiological benefits and/or reduces the risk of chronic diseases beyond basic nutritional functions (Health Canada, 1997). Apart from naturally occurring functional foods, such foods can be manufactured by the addition or enrichment of physiologically active components as ingredients (Erdmann *et al.*, 2008).

Flaxseed is one of the most prominent oilseeds studied today as a functional food besides soy. During the past decade, potential health benefits associated with flaxseed consumption have become more prominent. These health benefits include cardio protective effects, anticancer effects, antiviral and bactericidal activity, anti-inflammatory effect, iron reduction, laxative effects, beneficial effects on renal function in patients with lupus nephritis, mediation of bone health, management of diabetes, etc. (Cunnane *et al.*, 1995; Jenab & Thompson, 1996; Jenkins *et al.*, 1999; Kurzer & Xu, 1997; Oomah, 2001; Zhang *et al.*, 2008). Of these, there is an increasing interest in using flaxseed to reduce the risk of cardiovascular diseases (CVD). This is mainly based on the cardioprotective effects shown by bioactive components in flaxseed. There are human and animal studies reporting the cardioprotective mechanisms of flaxseed, which include hypolipidemic effects (Cunnane *et al.*, 1993; Cunnane *et al.*, 1995; Jenkins *et al.*, 1999), anti-inflammatory effects (Faintuch *et al.*, 2007), antiplatelet effects (Bierenbaum *et al.*, 1993) and antioxidant effects (Lee *et al.*, 2008). In a review of experimental and clinical research findings on the cardiovascular benefits of consuming flaxseed, Bassett *et al.* (2009) reported that consumption of 1-4 tablespoons of ground flaxseed per day can modestly reduce total cholesterol (TC; 6-11 %) and low density lipoprotein (LDL) cholesterol (9-18 %) levels and can reduce various markers

associated with atherosclerotic CVD in humans. The authors have also reported the inconsistency of findings regarding the antioxidative and antiplatelet effects of flaxseed in humans. The effects of flaxseed on arterial blood pressure have rarely been studied (Bassett *et al.*, 2009). Short term flaxseed consumption was found to exert no effect on arterial pressure of humans (Stuglin & Prasad, 2005). However, there are some human studies reporting a significant reduction in systolic and diastolic blood pressures (SBP and DBP) due to flaxseed (Dodin *et al.*, 2005) and flaxseed oil consumption (Paschos *et al.*, 2007). Moderate (30 g ground flaxseed per day) flax consumption has been reported to reduce atherosclerosis and irregular heart rhythms and reduce the risk of peripheral artery disease (PAD) (Dewan & Perkins, 2010).

2.5 Health claims for flaxseed in Canada

A health claim is considered to be any representation in labelling and advertising that states, suggests or implies that a relation exists between the consumption of foods or food constituents and health (Labbe *et al.*, 2008). Some food labels contain statements about the beneficial effects of certain foods on a person's health, such as "a healthy diet low in saturated and trans fat may reduce the risk of heart disease". This type of statement is an example of a health claim (Health Canada, 2010). There are three main categories of health claims allowed in Canada. They are;

1. Disease risk reduction claims: Links the consumption of foods or food constituents to a reduced risk of a disease in the context of the total diet
2. Function claims: Statements based on the specific beneficial effects that the consumption of a food or a food constituent has on the normal functions or biological activities of the body.

e.g. I Nutrient function claims: (formerly called biological role claims) are function claims that describe the well-established roles of energy or known nutrients that are essential for the maintenance of good health or for normal growth and development.

e.g. II Probiotic claims: are health claims about the benefits of probiotic microorganisms.

3. General health claims: are broad claims that promote health through healthy eating or that provide dietary guidance. These claims can be made on any product provided no linkage is made to a specific product or to a health effect, disease or health condition (Health Canada, 2010).

Health claims on foods impart benefits to stakeholders including regulatory bodies, industrial manufacturers, and consumers as well as to research institutions and scientists. For regulatory bodies, health claims provide a means to protect consumers from misleading or unsubstantiated information on product labels. For the food industry, health claims and nutrition labelling enable communication of health benefits of a food or food ingredient to consumers, which can also provide a strategic competitive advantage (Jew *et al.*, 2008). It has recently been suggested that consumers would be 10–26 % more likely to consume products with proven health claims, which may translate to a 20 % increase in sales. In turn, health claims tend to help consumers make better food choices and gain a deeper understanding of diet-disease relations (Jew *et al.*, 2008). Research institutions and scientists also benefit from conducting research on foods and their effects on health stimulated by the ability to develop and utilize health claims. In Canada, advertising health claims for foods is regulated under Food and Drug Regulations (FDR), whereas Health Canada develops the policies, regulations and standards related to health and safety (Labbe *et al.*, 2008). Substantiation of disease risk-reduction claims in Canada is based on the two key principles namely the totality of evidence and the requirement for a high or convincing level of evidence. First, a structured, comprehensive literature review of the totality of relevant evidence based on human studies of acceptable quality is required. Second, the strength of evidence must be convincing and consistently supports a causal relation between the consumption of foods or food constituents and the reduction of a disease risk (Labbe *et al.*, 2008).

Canadians are increasingly receptive to the idea of using food to prevent disease and improve health (Agriculture and Agri-Food Canada, 2010). Therefore, availability of health claims for food products are important to make consumers aware of their health benefits. Although there is research supporting the health benefits of flaxseed consumption, currently there are no approved health claims available for labelling flaxseed and their products in Canada. Small sample sizes, inconsistencies in products tested and other failures in study designs make study findings inconclusive. In a recent review, Arvanitoyannis and Houwelingen-Koukaliasoglou (2005) stated that research into functional foods will not advance public health, unless the benefits of the foods are effectively and clearly communicated to the consumer. Furthermore, foods with health claims that are supported by solid scientific substantiation have the potential to become increasingly important elements of a healthy lifestyle and can be extremely beneficial to the public and the food industry. When health claims are clearly supported by sound scientific evidence, consumers' confidence in them shall grow. Therefore, having health claims for flaxseed and their products will guide consumers on potential health benefits of flaxseed consumption, and will make flaxseed an appealing product in the functional food market increasing their market value. The FDA has approved qualified health claims for reduced risk of coronary heart disease (CHD) on conventional foods that contain ω -3 fatty acids (United States Food and Drug Administration, 2010). The approval of such health claims is expected to increase the use of ω -3 fatty acids containing foods such as flaxseed by health conscious consumers. Since flaxseed has gained GRAS status in the United States, Canada is now trying to develop health claims for flaxseed (Flax Council of Canada, 2010).

Sufficient conclusive scientific data that shows the relationship between flaxseed constituents and disease risk reduction is essential to obtain health claims for flaxseed products. The ample research publications available on cardioprotective functions of flaxseed constituents, especially ALA, lignan and mucilage are still controversial and inconsistent. Moreover, such information on flaxseed protein is scarce. Extensive, well designed studies on flaxseed constituents and heart health will provide necessary

information that will support setting health claims for flaxseed. Also it is of importance to identify the current information gap on flaxseed and heart health prior to focusing on human trials.

According to Health Canada (2010), health claims on foods would require a common scientific basis to provide consistent and credible messages to the public. It is important that any claimed benefit attributed to a product is justified by appropriate studies that are carried out based on sound scientific criteria. Although human studies are weighted most heavily in the evaluation of evidence on a substance/disease relationship, data from animal models and *in vitro* studies can also be used to support a substance/disease relationship. Animal and *in vitro* studies permit greater control over variables, such as diet and genetics, and permit more aggressive intervention. There is always an uncertainty when extrapolating data derived from these studies to physiological effects in humans. However, these studies can be useful in providing information on the mechanism of action and specificity of a food substance and the process that causes disease or health-related condition. Animal and *in vitro* studies should be considered when there are problems designing interventional studies or in the absence of an appropriate biomarker. If such studies are used, they are subjected to the same kind of assessment as human studies in assessing for health claims (United States Food and Drug Administration, 1999). Since human studies are more expensive to conduct, well designed *in vitro* studies that mimic human physiological condition can be used to generate supportive data on flaxseed and heart health.

2.6 Flaxseed components and reported cardiovascular benefits

Most of the studies on health attributes of flaxseed have focused on the bioactive constituents of flaxseed such as ALA in flaxseed oil, the lignan SDG and mucilage. Flaxseed is unique in its high content of the ω -3 fatty acid ALA, which is reported to have significant beneficial effects against heart disease. ALA can be converted in the body into cardioprotective ω -3-PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Harper *et al.*, 2006). The ω -3 fatty acids have been shown to regulate gene transcription and expression, thus altering enzyme synthesis and

to modify several risk factors for coronary heart disease including reducing serum triacylglycerols and blood pressure (Chen *et al.*, 2007; Dupasquier *et al.*, 2007; Waldschlager *et al.*, 2005). It has also been suggested that ALA could decrease blood pressure via its regulation of prostaglandin metabolism, which is involved in vascular reactivity, control of salt and water balance, regulation of renin release, effects on peripheral sympathetic tone, control of renal blood flow and effects on cardiac output and baroreceptors (Paschos *et al.*, 2007).

The anti-inflammatory, antioxidant, antiplatelet, hypolipidemic and hypotensive effects associated with flaxseed consumption were thought as consequences of ALA in flaxseed. However, in a rat feeding study, Bhathena *et al.*, (2003) showed that defatted flaxseed meal significantly decreased plasma cholesterol level compared to casein and reduced liver weight and hepatic fat deposition of obese rats compared to soy protein. The hypocholesterolemic action of defatted flaxseed meal also has been reported in human studies conducted by Jenkins *et al.*, (1999). Defatted flaxseed meal does not contain appreciable amounts of ALA. Therefore, these findings suggest that the hypolipidemic effect of flaxseed is not due to ALA. Recent studies on extracted flaxseed oil has further shown that there is no reduction in total cholesterol or LDL cholesterol levels in healthy humans upon consumption of flaxseed oil (Kaul *et al.*, 2008). Although there are animal studies supporting the fact that flaxseed oil is antithrombotic (Yamashita *et al.*, 2005) and inhibits platelet aggregation, there are human studies showing it has no antiplatelet activity (Vas Dias *et al.*, 1982). Partially defatted flaxseed meal has also shown antithrombotic and anti-atherogenic effect in mice (Sano *et al.*, 2003). Nestel *et al.* (1997) has reported no effect of flaxseed oil on blood pressure of humans.

The findings of studies on flaxseed suggest that there is a non-lipid component in flaxseed, which contributes to CVD protection. In a recent review, Bassett *et al.* (2009) have suggested that the hypocholesterolemic function of flaxseed is due to its dietary fibre and lignan content whereas flaxseed oil contributes to antiatherogenicity via anti-inflammatory and antiproliferative mechanisms. Prasad (2009) has conducted several

studies on flaxseed lignans and reported that suppression of atherosclerosis by flaxseed was associated with antioxidant activity and lowering of serum cholesterol, and is not due to its ALA but due to lignans. Based on animal and *in vitro* studies the author also suggested that flaxseed lignan and not ALA is contributing to the hypotensive activity of flaxseed. Also the hypotensive action of lignan was suggested to be mediated by the guanylate cyclase enzyme. Flaxseed lignans have chemical structures similar to the human hormone estrogen. When flaxseeds are ingested, SDG is converted to protective mammalian lignans, enterodiols and enterolactone, by the action of gut microflora (Chen *et al.*, 2003). The lignan complex isolated from flaxseed decreased oxidative stress and serum cholesterol in animal models (Prasad, 2005) and SDG isolated from flaxseed showed hydroxyl radical (OH[•]) scavenging activity *in vitro* (Prasad, 1997). However, studies done using mice have shown that lignans in defatted flax meal have no effect on atherogenesis or thrombosis (Sano *et al.*, 2003). Interestingly, there are recent human studies reporting that lignans isolated from flaxseed have no effect on plasma lipid concentrations, serum lipoprotein oxidation resistance, plasma antioxidant capacity (Hallund *et al.*, 2006) or endothelial function (Hallund *et al.*, 2006).

The controversies regarding the active component present in flaxseed, suggest that there has to be a non-lipid and non-lignan component in flaxseed that contributes to the cardioprotective function. The mucilaginous soluble fibre in flaxseed is reported to provide cardioprotective effects by reducing serum cholesterol and controlling hyperglycemia in humans, similar to guar gum, oat gum and other viscous fibre (Jenkins, 1995; Thakur *et al.*, 2009). In addition, it prevents development of colon and rectal cancer and reduces the incidence of obesity (Cunnane *et al.*, 1995; Oomah & Mazza, 1998). In the process of determining the phytochemicals with the most biological activity, some components of flaxseed, especially proteins, have received very little attention. At present, the official websites of flax organizations (Flax Council of Canada, 2010; Flax Canada 2015, 2010; and AmeriFlax, 2010) report cardioprotective benefits of flaxseed oil, lignans and fibre but not of proteins. This further shows the absence of published scientific evidence on flaxseed protein and CVD health. Oomah (2001) has pointed out that there is a lack of clinical studies conducted to

identify the bioactive potency of flaxseed gums and protein. This could be due to the understanding and belief that the secondary plant substances are the only bioactive phytochemicals. Although it has been reported that flaxseed mucilage may lower blood glucose, it is unlikely that the mucilage alone has such an effect. It has been observed that flaxseed protein may influence blood glucose by stimulating insulin secretion, which could result in reduced glycemic response (Nuttal *et al.*, 1984) and by interaction with polysaccharides. Lignans and other phenolics are also known to have strong protein-binding properties which may suggest some partial chemopreventive effect of flaxseed protein in conjunction with phenolics (Setchell *et al.*, 1983). At present there is evidence from epidemiological studies that suggests proteins from vegetables, and animal sources can prevent CVD (Zhang *et al.*, 2003). These research findings have motivated researchers to explore the role of flaxseed protein in the cardioprotective benefits exhibited by consumption of flax. To the best of my knowledge, human studies on health benefits of flaxseed protein, especially on cardiovascular health, is still in its infancy and have not yet been documented.

2.7 Flaxseed proteins

Flaxseed is a good source of protein. The protein content of flaxseed and defatted meal ranges from 10.5-31.0 % (Oomah & Mazza, 1993) and 35-40 % (Sammour, 1999), respectively and may vary with the genetic and environmental factors. Cool climates usually result in high oil and low protein content in the seeds. However, the genetic variation for protein content in flax cultivars is limited (Oomah & Mazza, 1998). While several studies have been conducted on extraction methods for flaxseed protein, only a few studies have characterized flaxseed proteins. Studies on isolating flaxseed protein were first reported by Osborne in 1892. Flaxseed has two major storage proteins, a predominant salt soluble fraction with high molecular weight (11-12S; globulin; 18.6 % nitrogen) and a water soluble basic component with low molecular weight (LMW) (1.6-2S; albumin; 17.7 % nitrogen) (Dev *et al.*, 1986; Madhusudhan & Singh, 1983; Sammour, 1999; Vassel & Nesbit, 1945). Globulins comprise 70-85 % of flaxseed proteins, two thirds of which has a molecular mass of 250 kDa. The remainder is of low molecular mass in nature (Madhusudhan & Singh, 1983). A reasonably well-balanced

amino acid composition has been reported for flaxseed protein (Sammour, 1999). In particular, it has a favorable ratio of amino acids with Lys, Thr and Tyr as the limiting amino acids. It is a good source of sulfur amino acids (Met and Cys) and is rich in branched chain amino acids (BCAA; Ile, Leu and Val) (Oomah & Mazza, 1995). Similar to other seed storage proteins (SSPs), the major protein of flaxseed (cultivar: NorMan) has high contents of the amino acids Arg, Glu/Gln, and Asp/Aspn (Oomah, 2001; Oomah & Mazza, 2000). The essential amino acid index of flaxseed meal is 69 compared to 79 and 75 for soybean and canola meals, respectively. Similarly, the protein score based on the most limiting amino acid relative to the Food and Agriculture Organization (FAO) requirement is 82 for flaxseed meal and 67 for soybean meal (Sosulski & Sarwar, 1973). The Lys to Arg ratio that determines the cholesterolemic and atherogenic effects of a protein (Czarnecki & Kritchevsky, 1992) is 0.37 for flaxseed, suggesting that it is less lipidemic and atherogenic compared to soybean or canola proteins, which have a Lys to Arg ratio of 0.88 (Oomah, 2001; Oomah & Mazza, 2000).

Although flaxseed proteins show similar properties to other oilseed proteins, the nutritional and therapeutic application of flaxseed protein has not yet been fully exploited. The present inconclusive findings on CVD protection by ALA and lignan in flaxseed is motivating a switch in research into flaxseed protein and investigation of whether the protein component of flaxseed is contributing to the cardioprotective benefits shown by flaxseed and its meal. However, upon consumption, protein undergoes digestion by a series of proteolytic enzymes in the GI tract. Therefore, it is important to investigate whether any of the products released during digestion of flaxseed protein is cardioprotective. In recent years, there has been a huge interest in the phenomenon of bioactive peptides. Apart from providing the amino acid requirement, food proteins including seed proteins have the ability to generate bioactive peptides possessing various physiological and health benefits (Kitts & Weiler, 2003). Since flaxseed protein is currently not considered as a bioactive component, studies on the ability of flaxseed proteins to release cardioprotective bioactive peptides during GI digestion would be of interest. Such studies would not only help to fill the present

information gap on health benefits of flaxseed protein but will support the regulatory bodies in the development of health claims for flaxseed and its products.

2.8 Food derived bioactive peptides

Bioactive peptides are defined as specific protein fragments that have a positive impact on body functions or conditions and which ultimately may influence health (Kitts & Weiler, 2003). They are inactive within the sequence of the parent protein, and can be released during hydrolysis by proteolytic microorganisms and through the action of proteolytic enzymes derived from microorganisms or plants (Korhonen & Pihlanto, 2006).

To date, controlled enzymatic hydrolysis is the most popular method to produce bioactive peptides from food proteins. Although peptides can be released from acid hydrolysis, which is simple and less expensive, enzymatic hydrolysis is easier to control and does not damage the amino acids due to the use of mild reaction conditions (Gilani *et al.*, 2008). Proteinases (endopeptidases) such as chymotrypsin, papain, pepsin, plasmin, proteinase K, subtilisin, Thermolysin, and trypsin, are some of the commonly used enzymes for the production of bioactive peptides from food protein (Yamamoto *et al.*, 2003).

Gastrointestinal digestion is another important pathway by which bioactive peptides are released from food proteins. The bioactive peptides generated during GI digestion of food proteins as well as orally administered bioactive peptides following digestion, can either be absorbed via the intestine, enter the blood circulation intact and exert systemic effects or produce local effects in the GI tract (e.g. by binding directly to epithelial cell surface receptor sites). The main mechanisms in the human body by which these bioactive peptides can be activated or inactivated include digestion by proteolytic digestive enzymes, absorption through the intestinal tract and further hydrolysis by serum peptidases (Pihlanto & Korhonen, 2003). Bioactive peptides have been shown to possess different activities *in vitro* and *in vivo* depending on the sequence of amino acids. Such activities include antihypertensive, anti-oxidative, antithrombotic,

antimicrobial, hypocholesterolemic, opioid, immunomodulatory and other activities. (Erdmann *et al.*, 2008; Pihlanto & Korhonen, 2003; Yust *et al.*, 2003). Many of the bioactive peptides are multifunctional and can possess more than one activity. Upon oral administration, bioactive peptides may affect the major body systems, namely the cardiovascular, digestive, immune and nervous systems, reducing the risk of various lifestyle related diseases (Korhonen & Pihlanto, 2006; Yoshikawa *et al.*, 2000). Due to the health enhancing properties and safety profiles such as absence of toxicity, these peptides have gained interest as components in functional foods and nutraceuticals. The most widely used method for yielding bioactive peptides *in vitro* is the enzymatic hydrolysis by pancreatic enzymes, especially trypsin. Once the amino acid sequence of the peptide is known, the peptides can be synthesized by chemical synthesis or recombinant DNA technology. Chemical synthesis is useful for synthesis of short sequences whereas the latter is useful for larger peptides (Moller *et al.*, 2008).

Food-derived bioactive peptides commonly contain two to nine amino acids (Kitts & Weiler, 2003). However, this range may be extended to 3-20 or more amino acid residues (Korhonen & Pihlanto, 2003). For example lunasin, a food-derived peptide with proven anticancer activity, contains 43 amino acids with a molecular weight of 5400 Da (Joeng *et al.*, 2002). Bioactive peptides have been mainly isolated and studied from animal protein sources including bovine milk, cheese and dairy products. (Moller *et al.*, 2008). Bioactive peptide sequences are found in other animal and plant proteins too. These include bovine blood, gelatin, meat, eggs, fish, wheat, soy, corn, rice, sorghum, mushroom, pumpkin and garlic (Darragh, 2002; Moller *et al.*, 2008). Several food applications of bioactive peptides, especially those with antihypertensive activity, have already evolved; caseinophosphopeptides are currently used as dietary and pharmaceutical supplements, and fermented milk products based on antihypertensive peptides are on the market in Japan and Finland (Korhonen, 2002; Yamamoto, 2010). Antihypertensive peptides prepared from hydrolysis of sardine protein and peptide soups containing antihypertensive bonito hydrolysate are currently available in Japan (Yamamoto, 2010). However, most of the exogenous bioactive peptides are ineffective after oral administration because of their sensitivity to endothelial peptidases or

difficulty in intestinal absorption. In contrast, some bioactive peptides derived from food proteins are active after oral administration even though their specific activities are not very high. This is due to their smaller molecular size and resistance to peptidases (Yoshikawa *et al.*, 2000). However, research on the absorption of biologically functional peptides and digestive enzyme resistant peptides in the intestine is limited (Yamamoto *et al.*, 2003).

2.9 Fate of food proteins and bioactive peptides in the human gastrointestinal tract

Food proteins are extensively hydrolyzed at all stages of GI digestion before being transferred as di- or tri-peptides to the basolateral side of the intestinal enterocytes (Roberts *et al.*, 1999). The human GI tract secretes a number of peptidases that function synergistically to cleave polypeptide chains into amino acids and small peptides. It is evident that a significant proportion of dietary nitrogen is absorbed into the body in the form of small peptides (Roberts *et al.*, 1999). Due to their specific activities, digestive enzymes play a major role in regulating the release of bioactive peptides from food proteins. Therefore, the digestibility of a food protein is an indication of its efficacy to generate bioactive peptides during GI digestion. Bioavailability is usually defined as the fraction of an oral dose of a parent compound or active metabolite from a particular preparation that reaches the systemic circulation (Fernandez-Garcia *et al.*, 2009). Therefore, when a food protein is ingested, its digestibility as well as bioavailability of the end products will play crucial roles in determining whether bioactive peptides can be released from the protein and become available to express respective bioactivities in the human body. The physical form in which foods are consumed is considered as an important factor to be considered in determining the digestibility and bioavailability of nutrients (Bjorck *et al.*, 1994).

The food proteins are exposed to various proteolytic enzymes during their passage through GI tract. The ingested food, after mastication and bolus formation in the mouth, passes into the stomach where the pH is in the range of 1.5-3. The digestion of food proteins begins in the stomach where proteolysis is initiated by pepsins that are activated at acidic pH. The pepsins catalyze hydrolysis of peptide bonds involving Phe,

Tyr and Leu (Erickson & Kim, 1990). This gastric phase results in a mixture of long polypeptides, short oligopeptides, and some free amino acids (Erickson & Kim, 1990). When the digested products enter into the first part of the small intestine, the duodenum, the products are further digested by enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and B) that are synthesized and released by the pancreas. The pancreatic enzymes are activated in the duodenum where the rise in pH to neutral or slightly alkaline conditions inactivates the gastric pepsins. Trypsin cleaves the peptide bonds on the carboxyl side of the basic amino acids (Arg, Lys). Chymotrypsin hydrolyzes peptide bonds where the carbonyl groups are aromatic (Tyr, Phe and Trp). Elastases are endopeptidases that cleave the interior peptide bonds of protein and polypeptides. Elastase hydrolyzes peptide bonds where the amino acid contributing to the carboxylic groups are aliphatic (Ala, Leu, Gly, Val, Ile). Carboxypeptidase A and B are major exopeptidases in pancreatic secretion. They remove single amino acid groups from the carboxylic ends of proteins and oligopeptides. These pancreatic enzymes digest the peptides released from the gastric phase further into a mixture of short oligopeptides (2-6 amino acids) and free amino acids (Erickson & Kim, 1990).

In the small intestine, the proteins are exposed to an extensive range of biosurfactants including bile acids and phospholipids and the products of lipolysis. Although bile acids aid fat digestion, recent studies show that they are important for protein digestion as well. Gass *et al.* (2007) showed that hydrolysis of proteins by trypsin and chymotrypsin was significantly enhanced by the presence of conjugated bile acids. It was suggested that this was due to the bile acids destabilizing the protein structure and making it more susceptible to proteolysis. However, this was not shown for all proteins and the effect of bile acids is attenuated in the presence of high levels of fatty acids and monoglycerides as the competition for bile acids to get incorporated into the fat based micelles is increased.

The small intestine is the principal site of absorption of the end products of protein digestion. It is shown that small (di- and tri-peptides) and large (10-51 amino acids) peptides generated in the diet can be absorbed intact through the intestine and produce

biological effects at the tissue level (Roberts *et al.*, 1999). The free amino acids are absorbed via enterocytes of the small intestinal brush border membrane through distinct amino acid transport systems. However, the oligopeptides can undergo further hydrolysis prior to absorption by brush border peptidases resulting in a mixture of free amino acids, di and tri peptides. The intestinal brush border is rich in amino peptidases, which provide functional complementation to carboxypeptidases in pancreatic juice. The major amino peptidases (N and A) cleave N terminal neutral and anionic amino acids, respectively. In addition, the intestinal brush border has endopeptidase and dipeptidase activity. For example dipeptidyl amino peptidase IV releases dipeptides from the N-terminus of oligopeptides with Pro or Ala in the penultimate position. Hence, the dipeptides released by the brush border enzymes are generally of the X-Pro type (Vermeirssen *et al.*, 2004). Proline and hydroxyl-Pro-containing peptides are generally resistant to degradation by these brush border enzymes. Furthermore, tripeptides containing the C-terminal Pro-Pro are reported to be resistant to Pro-specific peptidases. Also it has been observed that large polypeptides or proteolysis-resistant entire proteins can enter the blood circulation in small amounts (Vermeirssen *et al.*, 2004).

There are several mechanisms by which peptides are transported through the intestinal epithelium. Electrophysiological studies conducted during 1970s and 1980s have suggested the existence of a peptide transport system in the intestinal epithelium by which peptides would be actively transported through the intestinal membrane (trans cellular) under a H^+ gradient (Ganapathy & Leibach, 1985). However, this transport mechanism carries only di- and tri-peptides (Daniel *et al.*, 1992) whereas oligopeptides with more than four residues are transported through the intestinal epithelium via other routes such as pinocytosis or through paracellular channels (Roberts *et al.*, 1999) depending on the molecular size and hydrophobicity. Figure 2.1 provides a schematic overview of these two pathways. After absorption via the intestinal tract, serum peptidase can further hydrolyze the peptide bonds. Therefore, resistance to peptidase degradation is a prerequisite for physiological effects of bioactive peptides following oral ingestion or intravenous infusion.

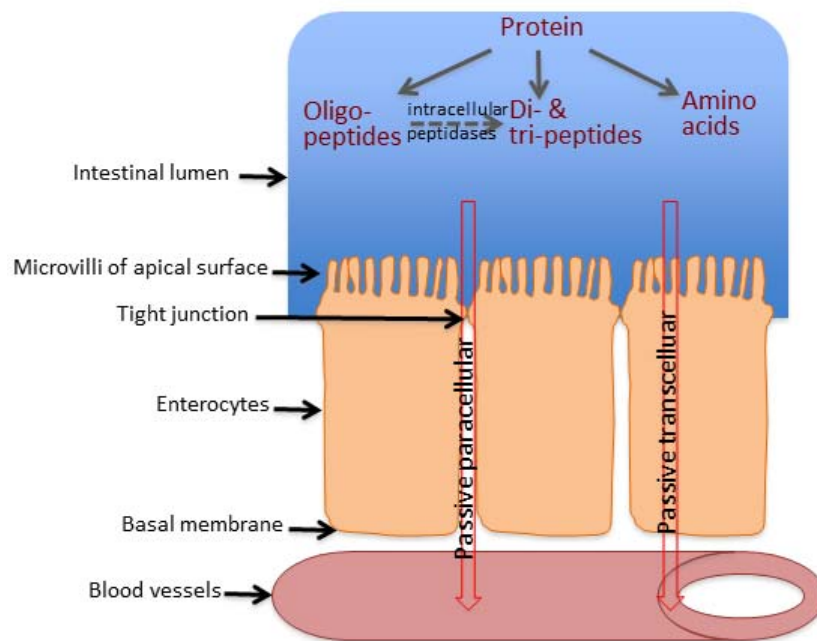


Figure 2.1 Schematic explanation of potential paths of peptide and amino acid absorption in the small intestine

2.10 Use of *in vitro* methods to assess protein digestibility and release of peptides

The amounts of essential amino acids in proteins, the digestibility, the ability to release amino acids and peptides with biological activities during digestion and the bioavailability of the digested products are among the main determinants of the nutritional quality and health benefits of food proteins (Gauthier *et al.*, 1986). Knowledge of the release of intermediate peptides, amino acid composition and size distribution of peptides released during protein digestion is essential for explaining their biological actions (Agudelo *et al.*, 2004). Therefore, investigations on digestion of food proteins and absorption of digested products in humans are important to understand the physiological and nutritional roles of proteins. However, due to ethical reasons and the cost involved, it is becoming increasingly difficult to carry out such research using humans as subjects. Hence, there is a need for exploring validated animal models and *in vitro* models, which can adequately predict protein digestion and absorption in humans.

Pigs and rats have been widely used as animal models. *In vitro* studies have been developed to simulate the physiological conditions and sequence of events that occur during digestion in the human GI tract. The features that simulate *in vitro* models to true GI digestion are physiological temperature, mixing or agitation, chemical and enzymatic composition of saliva, gastric juice, duodenal juice and bile juice. The *in vitro* GI model is defined as a static model when physical processes that occur *in vivo* are not reproduced. The dynamic models mimic the *in vivo* physical processes. They take into account new variables such as changes in the viscosity of digesta, particle size reduction, diffusion and partitioning of nutrients (Fernandez-Garcia *et al.*, 2009).

A more practical and realistic approach to *in vitro* models is to separately evaluate the specific contributions of oral and gastric digestion, intestinal digestion by pancreatic enzymes, brush-border hydrolysis, and eventually intestinal absorption and enterocyte metabolism (Savoie, 1994). In the commonly used two stage *in vitro* technique, the protein substrate is first digested with pepsin and then by pancreatin. Pancreatin is a crude preparation from pancreatic secretions that includes proteases such as trypsin and chymotrypsin (Qiao *et al.*, 2005). However, when such enzymatic hydrolysis occurs in a batch process, accumulation of hydrolysis products does not reflect what happens in the GI tract. The accumulation of the proteolytic reaction products may impede the progression of hydrolysis (Stahmann & Woldegiorgis, 1975). Therefore, continuous removal of digestion products during *in vitro* proteolysis represents a more realistic model that represent *in vivo* mechanisms, where the epithelial brush border membranes remove the products of protein digestion from the lumen by independent mechanisms (Savoie, 1994).

The two-step proteolysis model (digestion cell) developed by Savoie and Gauthier (1986) closely simulates the *in vivo* digestion process. This includes digestion of protein with a pepsin-containing reaction mixture in a closed system followed by digestion with pancreatin in a dialysis tube of 1 kDa molecular weight cut off. The digestion cell apparatus developed to perform the pancreatic digestion is made up of two concentric compartments. The enzymatic reaction takes place in the inner cylinder surrounded by

the membrane and mixing is provided by a magnetic stirrer. The products released during digestion diffuse through the membrane and are collected by the circulation of sodium phosphate buffer in the outer cylinder. The entire system is submerged in a water bath maintained at constant temperature. Simultaneous removal of hydrolysis products is achieved by continuous replacing of the buffer using a peristaltic pump. This approach enables direct identification of amino acid composition of the digestion products and their release kinetics during the digestion process. Also it allows for continuous collection of dialyzable digestion products of low molecular weight (less than 1 kDa) that correspond to the end products of luminal digestion (Agudelo *et al.*, 2004). However, it is important that an *in vitro* versus *in vivo* validation process is carried out to delineate the reliability of the *in vitro* models.

2.11 Role of food derived bioactive peptides in reducing cardiovascular diseases

Cardiovascular disease is the leading cause of death worldwide. In 2004 an estimated 17.1 million people died from CVDs, representing 29 % of all global deaths. Of these deaths, an estimated 7.2 million were due to CHD and 5.7 million were due to stroke (World Health Organization, 2009).

There are various factors leading to CVD, which are called CVD risk factors. In order to prevent CVD it is important to identify the major modifiable risk factors. They include tobacco and alcohol use, high blood pressure (hypertension), high serum low density lipoprotein (LDL) cholesterol, elevated glucose, obesity, physical inactivity, and unhealthy diets (MacKay & Menash, 2004; Smith *et al.*, 2004). Since diet is a significant modifiable risk factor for CVD (World Heart Federation, 2007), choosing healthy food plays a major role as it can reduce the occurrence of many of the listed modifiable CVD risk factors. The current dietary recommendations to reduce CVD risk encourages consumption of diets rich in vegetables, fruits, whole grains and high fibre foods, whereas consumption of oily fish at least twice a week is recommended. It is also recommended to reduce saturated fat (<7 % energy) and trans fat (<1 % energy) in the diet and minimize consumption of foods with added sugars and salt in order to reduce the risk of CVD (Lichtenstein *et al.*, 2006). Moreover, there is research evidence to

suggest that an increased consumption of protein (e.g. replacing carbohydrates in diet with protein), particularly plant protein, may further lower the risk of hypertension and CVD (Hu *et al.*, 1999; Stamler *et al.*, 1999). Release of cardio protective bioactive peptides upon dietary protein ingestion could be the mechanism behind these findings. Major activities of bioactive peptides that are related to CVD health are discussed below.

2.12 Bioactivities of food derived peptides

2.12.1 Antihypertensive effect and angiotensin I-converting enzyme inhibitory peptides

Hypertension or high blood pressure is a major health problem all over the world, affecting 50 % of individuals above 55 years in many industrialized countries, and is a major risk factor for coronary heart disease, congestive heart failure, stroke and renal disease (Hermanson, 2000). A person is said to be hypertensive when SBP >140 mmHg or DBP >90 mmHg (140/90 mmHg) or when taking medications for hypertension (Wolz *et al.*, 2000). In a recent meta-analysis of clinical trials, Pripp (2008) identified that food protein derived peptides play a significant role in blood pressure reduction. Many of these clinical trials were done using ACEI peptides.

Angiotensin I-converting enzyme plays a critical role in regulation of blood pressure. It is a zinc metalloprotease, a component in the renin angiotensin system (RAS), and a dipeptidylcarboxypeptidase that hydrolyzes carboxy terminal dipeptides from oligopeptide substrates (Figure 2.2). ACE and other components of RAS coexist locally in various tissues including blood vessels, kidney, adrenal glands, heart and brain in addition to circulation. High concentrations of ACE are found on epithelial surfaces such as intestinal, pulmonary surface, choroid plexus, and placental brush borders influencing the fluid and electrolyte balance at these fluid membrane interfaces (Ehlers & Riordan, 1989). ACE increases blood pressure by catalyzing the conversion of the decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II, and also by catalyzing the degradation of bradykinin, a blood pressure lowering nonapeptide.

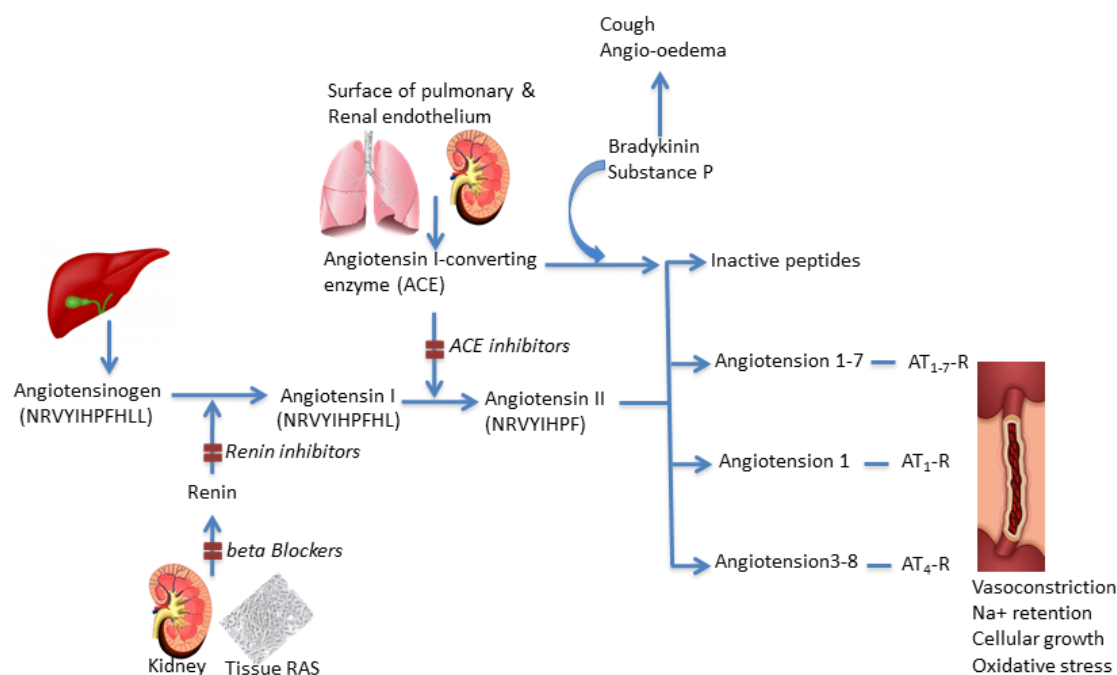


Figure 2.2 Summary of renin-angiotensin system indicating major drug inventions (ACE inhibitors, Beta blockers and Renin inhibitors) to regulate hypertension

Angiotensin II leads to several effects that are central and causes further increases in blood pressure (Figure 2.2). Therefore, inhibition of ACE results in an overall antihypertensive effect (Vermeirssen *et al.*, 2002). ACE has two domains (N and C), each of which contains an active site with a His-Glu-X-X-His. The His residues are considered to participate in Zn binding and Glu residue in the catalytic mechanism. Of these, the C domain is the dominant angiotensin I-converting site (Natesh *et al.*, 2003). The first available competitive inhibitors of ACE were isolated from venom of *Bothrops jararaca* snake as naturally occurring peptides (Ferreira *et al.*, 1970). The pentapeptide, Glu-Lys-Trp-Ala-Pro and the nonapeptide Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro isolated from the venom were found to have antihypertensive properties.

There are studies indicating the effect of peptide structure on its ACEI activity. The structure-activity correlation studies of analogs of both peptides indicated that their

carboxy terminal tripeptide residues (Trp-Ala-Pro) play a predominant role in competitive binding to the active site of ACE (Cheung *et al.*, 1980) (Figure 2.3). The antihypertensive effect of such peptides led to development of the highly potent ACEI drug, Captopril that inhibits ACE by coordinating strongly with the Zn atom in the active site of ACE by coupling its sulfhydryl group to the dipeptide Ala-Pro (Figure 2.3).

Numerous other ACE inhibitors such as Enalapril[®] and Lisinopril[®] have also been developed (Wei *et al.*, 1992). In addition to ACE inhibitors, renin inhibitors, which block the action of renin and thereby prevent formation of both angiotensin I and angiotensin II, also have been developed for lowering blood pressure. Renin inhibitors do not affect kinin metabolism and are not expected to cause dry cough and angioneurotic edema, which are characteristic side effects of ACE inhibitors (Karlberg, 1993). However, except for Alliskerin (a non-peptide renin inhibitor), most of the renin inhibitors identified so far were found to be clinically ineffective when given orally (Gradman *et al.*, 2005).

Several studies have been conducted to identify and isolate peptides with ACEI activity from food proteins. Trp, Tyr, Phe, and Pro are the most potent C-terminal amino acids of these peptides that contribute immensely to substrate binding at the ACE active site and thereby inhibit their activity of ACE (Cheung *et al.*, 1980). Wu *et al.* (2006), from a database containing 168 dipeptides and 140 tripeptides, identified that amino acid residues with bulky side chains as well as hydrophobic side chains are preferred for ACEI dipeptides. For tripeptides, the most favourable residues for the carboxyl terminus are aromatic amino acids, while positively charged amino acids are preferred for the middle position, and hydrophobic amino acids for the amino terminus.

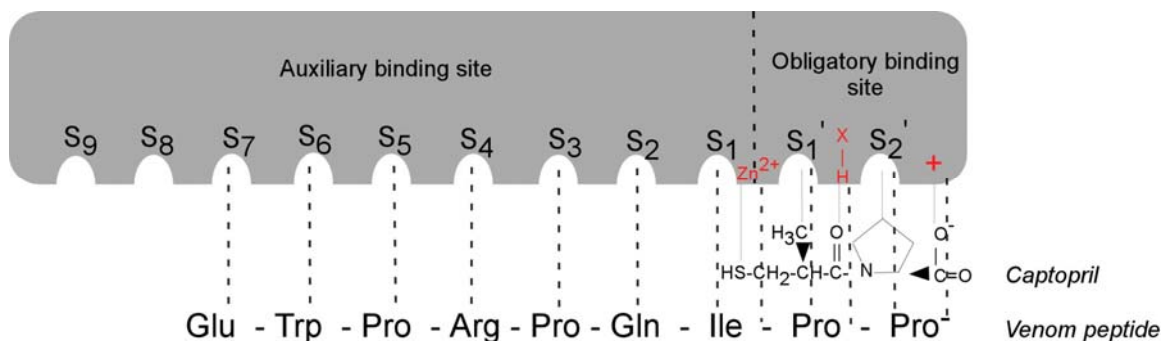


Figure 2.3 Proposed model for binding of venom nonapeptide and Captopril on to the active sites of angiotensin I-converting enzyme (Modified from Cushman *et al.*, 1981)

Recently, few studies have shown the presence of ACEI activity in hydrolyzed flaxseed protein (Wu *et al.*, 2004; Udenigwe *et al.*, 2009). ACEI peptide sequences can be liberated from flaxseed protein by proteolytic enzymes as demonstrated with defatted flaxseed meal in a recent patent (Wu *et al.*, 2004). Recently, Udenigwe *et al.* (2009) hydrolyzed flaxseed protein using various proteases (alcalase, ficin, pancreatin, papain, pepsin, Thermolysin and trypsin) and the resulting hydrolysates were tested for ACEI and renin inhibitory activity. In this study, all the protein hydrolysates showed ACEI activity whereas <1 kDa fraction of papain and pancreatin hydrolysate did not show renin inhibition. However, it is still not known whether these ACEI peptides can be released during sequential digestion of flaxseed protein by GI enzymes.

Table 2.1 Examples of angiotensin I-converting enzyme inhibitory peptides derived from enzymatic hydrolysis of seed storage proteins

Source of protein	Enzymes generating ACEI peptides	Amino acid sequence of peptides	Details	Reference
Wheat (<i>Triticum aestivum</i> L.) germ	<i>Bacillus licheniformis</i> alkaline protease	Ile-Val-Tyr	Simulated GI digestion increased the activity	Matsui <i>et al.</i> , 1999
Buckwheat (<i>Fagopyrum esculentum</i> Moench) flour	Pepsin, chymotrypsin & trypsin	Tyr-Gln-Tyr Pro-Ser-Tyr	Lowered SBP of SHR	Li <i>et al.</i> , 2002
Chick pea (<i>Cicer arietinum</i> L.) legumin	Alcalase	Met-Asp, Asp-Phe-Leu-Ile, Met-Phe-Asp-Leu Met-Asp-Leu Met-Asp-Leu-Ala	<i>In vitro</i> study	Yust <i>et al.</i> , 2003
Field pea (<i>Pisum sativum</i> L.) protein	pepsin, chymotrypsin & trypsin	NR	Lowered mean arterial blood pressure in SHR	Vermeirssen <i>et al.</i> , 2005
Mung bean (<i>Phaseolus radiatus</i> L.) protein	Alcalase	Lys-Asp-Tyr-Arg-Leu Val-Thr-Pro-Ala-Leu-Arg Lys-Leu-Pro-Ala-Gly-Thr-Leu-Phe	<i>In vitro</i> study	Li <i>et al.</i> , 2006
Soybean (<i>Glycine max</i> L. Merr.)	Alcalase	Asp-Leu-Pro, Asp-Gly	Stable to <i>in vitro</i> GI digestion	Wu & Ding, 2002
	Pepsin	Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, Ile-Tyr-Leu-Leu	Lowered blood pressure in SHR	Chen <i>et al.</i> , 2003 Cont'd.

Table 2.1 contd. Examples of angiotensin I-converting enzyme inhibitory peptides derived from enzymatic hydrolysis of seed storage proteins

Source	Enzymes generating ACEI peptides	Amino acid sequence of peptides	Details	Reference
Soybean (<i>Glycine max</i> L. Merr.)	Pepsin & pancreatin	NR	<i>In vitro</i> dynamic model simulating upper GI tract	Lo <i>et al.</i> , 2006
Rapeseed/Canola (<i>Brassica napus</i>) protein	Subtilisin [®]	Ile-Ty, Val-Trp, Val-Trp-Ile-Ser	Lowered blood pressure in SHR	Marczak <i>et al.</i> , 2003
Canola meal	Alcalase	Val-Ser-Val Phe-Leu	<i>In vitro</i> study	Wu <i>et al.</i> , 2008
Sunflower (<i>Helianthus ananus</i> L.) protein	Pepsin and pancreatin	Phe-Val-Asn-Pro-Gln-Ala-Gly-Ser	<i>In vitro</i> study	Megias <i>et al.</i> , 2004
Flaxseed protein	Pepsin, ficin, trypsin, papain, Thermolysin, pancreatin and Alcalase	NR	<i>In vitro</i> study	Udenigwe <i>et al.</i> , 2009

NR: Not reported; SBP: Systolic blood pressure; SHR: Spontaneously hypertensive rats; GI: Gastrointestinal

2.12.2 Antioxidant activity

Free radicals such as superoxide ($O_2^{\bullet-}$), OH^{\bullet} and other oxygen-derived species (H_2O_2 , and hypochlorous acid) are formed constantly in the human body during oxygen metabolism in addition to exposure to oxidants such as air pollutants, ozone, oxides of nitrogen, tobacco smoke, and motor vehicle exhaust (Clarkson & Thompson, 2000). The free radicals are defined as chemical species that contain an unpaired electron in the outer orbital (Cheesman & Slatter, 1993). They are highly reactive as the unpaired electron in the outer orbit allows attraction of electrons from other molecules resulting in another free radical that is capable of reacting and damaging another molecule (Clarkson & Thompson, 2000). Molecules such as hydrogen peroxide (H_2O_2) that are generated in the biological system via free radicals are reactive oxygen species (ROS). The ROS includes both oxygen derived free radicals and non-radical oxygen derivatives that are involved in oxygen radical production (Cheeseman & Slatter, 1993). For example, $O_2^{\bullet-}$ arising either through metabolic processes or following oxygen “activation” by physical irradiation, is considered the “primary” ROS. Two $O_2^{\bullet-}$ molecules can react together to form hydrogen peroxide and oxygen. H_2O_2 can break down to generate the most reactive OH^{\bullet} via the Haber-Weiss reaction which proceeds quickly when metal ions such as Fe^{2+} or Cu^+ are present (Fenton reaction when iron is the catalyst) (Benzie, 2000; Cheeseman & Slatter, 1993). The free radicals can attack all macromolecules, causing lipid peroxidation (oxidative destruction of PUFAs in cell membranes), DNA damage and protein degradation during oxidatively stressful events. Of these, unsaturated lipids are the most susceptible (Clarkson & Thompson, 2000). Specifically, the oxidative modification of LDL leads to oxidized LDL that can increase atherogenesis. Therefore, uncontrolled production of free radicals is associated with the onset of various CVDs including atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Clarkson & Thompson, 2000).

The human body has an endogenous antioxidant defense system that consists of various enzymes (such as catalase and superoxide dismutase) and antioxidant compounds. These antioxidants can prevent or inhibit oxidation by preventing generation or by inactivation of ROS. Several naturally occurring antioxidant peptides such as glutathione (GSH: Glu-Cys-Gly), carnosine (β -Ala-L-His), anserine (β -Ala-3 methyl-L-His), homocarnosine (γ aminobutyryl-L-His) with free radical scavenging activity have been identified in the human body (Kelly, 1999). Of these, GSH scavenges free radicals by donating its H atom and by acting as a co-substrate in reduction of H_2O_2 and other hydroperoxides by GSH peroxidase (Kelly, 1999). Amongst the His-containing peptides, carnosine, one of the most abundant (1-20 mM) nitrogenous compounds present in the non-protein fraction of vertebrate skeletal muscle and certain other tissues, including olfactory epithelium, scavenges OH^\bullet , O_2^\bullet and chelates Cu, which in turn prevents the Haber-Weiss reaction (Gariballa & Sinclair, 2000). However, these endogenous defenses are not completely efficient (Jacob & Burry, 1996). When ROS are generated excessively or the antioxidative defenses are depressed (oxidative stress), a number of pathological events take place (Figure 2.4). Therefore, antioxidants in the human diet are of great interest as possible protective agents to help the human body to reduce oxidative damage (Wu *et al.*, 2005).

In addition to the well-known dietary antioxidants such as vitamin C, E, polyphenols and carotenoids, recent studies have shown that peptides with antioxidant activity can be released by food proteins. Some of these food derived antioxidant peptides exert antioxidant activity against enzyme (lipoxygenase mediated) and non-enzyme peroxidation of lipids and essential fatty acids (Chen *et al.*, 1996). The antioxidant mechanism of these peptides has been suggested to be due to metal ion chelation, free radical scavenging and singlet oxygen quenching or a combination of these mechanisms (Chen *et al.*, 1998; Kitts & Weiller, 2003). Another possible mechanism could be induction of genes that protect cells from ROS damage (Erdmann *et al.*, 2008). The major examples of food proteins releasing antioxidant peptides are milk casein (Suetsuna *et al.*, 2000), whey protein (Hernandez-Ledesma *et al.*, 2005) and egg proteins (Davalos *et al.*, 2004).

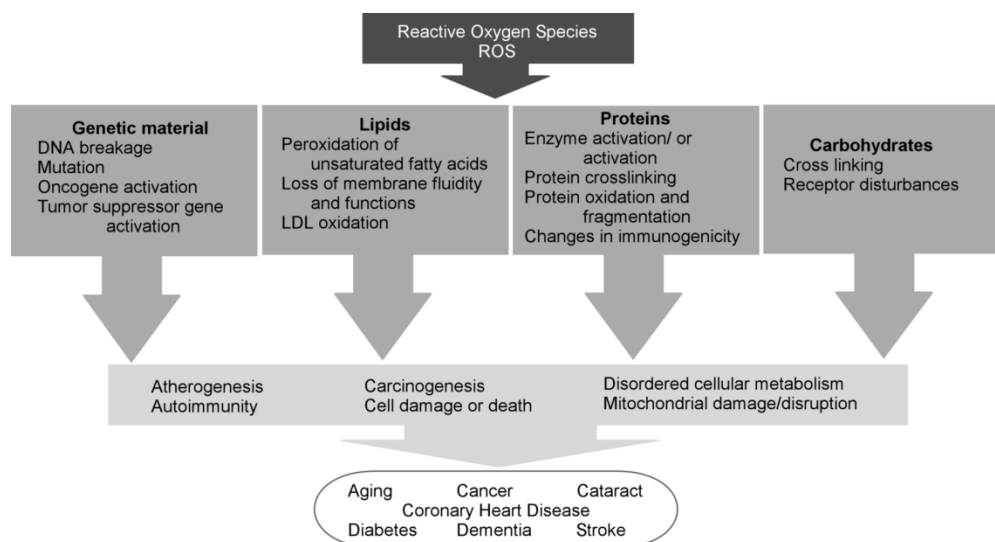


Figure 2.4 Damage to biological molecules by reactive oxygen species leading to increased risk of diseases (Modified from Benzie, 2000)

Several studies report the antioxidant activities of food protein hydrolysates *in vitro* operating on multiple antioxidant mechanisms (Table 2.2). The majority of hydrolysates have shown multiple antioxidant mechanisms (Table 2.2). However, only few studies have attempted to isolate and sequence the antioxidant peptides from the hydrolysates. Such studies show the importance of certain amino acids in the peptide sequence to exert the antioxidant mechanism. Chen *et al.* (1995) isolated six antioxidant peptide fragments from digestion of soy β conglycinin with protease S. The peptides (P1: Val-Asn-Pro-His-Asp-His-Gln-Asn; P2: Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn, P3: Leu-Leu-Pro-His-His, P4: Leu-Leu-Pro-His-His-Ala-Asp-Ala-Asp-Tyr, P5: Val-Ile-Pro-Ala-Gly-Tyr-Pro, P6: Leu-Gln-Ser-Gly-Asp-Ala-Leu-Arg-Val-Pro-Ser-Gly-Thr-Thr-Tyr) consisted of 5-16 amino acid residues including hydrophobic amino acids such as Val or Leu at the N-terminal position and Pro, His and Tyr in the sequences. Suetsuna *et al.* (2000) isolated $O_2^{\bullet-}$, OH^{\bullet} and DPPH radical scavenging peptide Tyr-Phe-Tyr-Pro-Glu-Leu from pepsin hydrolyzed casein and observed that the Glu-Leu analog of the peptide plays a role in free radical scavenging.

Table 2.2 Antioxidant mechanisms of selected food protein hydrolysates

Source protein	Enzyme/s	Antioxidant Mechanism	Reference
Casein	pepsin	Scavenging DPPH [•] , O ₂ ^{•-} , OH [•]	Suetsuna <i>et al.</i> , 2000
Soy β conglycinin	microbial enzymes (Protease M, N, P and S) and Pepsin	Inhibition of linoleic acid peroxidation	Chen <i>et al.</i> , 1995
Wheat germ protein isolate	Alcalase	Inhibition of linoleic acid peroxidation, reducing power, Fe ²⁺ chelating effect, scavenging DPPH [•] , O ₂ ^{•-} , OH [•]	Zhu <i>et al.</i> , 2006
Zein from maize	Alcalase and papain	Inhibition of liposome oxidation, Cu ⁺ chelation, reducing power, radical scavenging	Kong & Xiong, 2006
Chick pea protein	Alcalase	Inhibition of linoleic acid oxidation, Reducing power, Scavenging DPPH [•] , O ₂ ^{•-} , OH [•]	Li <i>et al.</i> , 2008
Rapeseed protein	Alcalase	Reducing power, Scavenging DPPH [•] , O ₂ ^{•-} , OH [•]	Pan <i>et al.</i> , 2009
Lima bean (<i>Phaseolus lunatus</i>) and jamapa bean (<i>Phaseolus vulgaris</i>) protein	Alcalase and Flavourzyme	Scavenging ABTS ^{•+} radical cation	Torruco-uco <i>et al.</i> , 2009

O₂^{•-}: Superoxide radical; OH[•]: Hydroxyl radical; DPPH[•]: 2, 2-diphenyl-1-picrylhydrazyl radical; ABTS^{•+}: 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

The exact mechanism by which peptides exhibit antioxidant potency is not clear. However, several research findings show that amino acid composition and structural features of the peptide play a role. In a recent review, Erdmann *et al.* (2008) reported that the presence of a high amount of His and some hydrophobic amino acids in the peptide is related to their antioxidant potency. The activity of His-containing peptides is thought to be due to hydrogen donating ability, lipid peroxy radical trapping, and /or metal ion chelating ability of the imidazole group (Erdmann *et al.*, 2008). Also the addition of Leu or Pro residues to the N-terminus of a His-His peptide would enhance antioxidant activity. Chen *et al.* (1996) have shown that a soy protein derived peptide with a Pro-His-His sequence shows the highest antioxidant capacity. The hydrophobicity of the peptide also would play a role due to its increased accessibility to hydrophobic targets such as fatty acids (Chen *et al.*, 1998). Furthermore, it is evident that the antioxidant effect of certain amino acids are higher when they are incorporated into dipeptides suggesting that the peptide bond and specific structural features of the peptides also play a crucial role in determining antioxidant potency (Erdmann *et al.*, 2008).

Udenigwe *et al.* (2009) recently reported on the ability of isolated flaxseed protein to liberate antioxidant peptides (with DPPH[•], O₂^{•-}, OH[•]) when hydrolyzed with ficin, pancreatin, papain, pepsin, Thermolysin and trypsin. These finding support our hypothesis that flaxseed protein consist of amino acid sequences that have antioxidant properties. However, whether flaxseed protein can still release these antioxidant peptides during the sequential digestion of protein in the GI tract is not known yet.

2.12.3 Cholesterol lowering ability

Among the dietary proteins, soy protein, has demonstrated cholesterol lowering properties in various populations (D'Amico *et al.*, 1992; Laurin *et al.*, 1991). In a meta-analysis of human studies, Anderson *et al.* (1995) showed that rather than animal proteins, soy protein intake significantly decreased serum concentrations of total cholesterol, LDL cholesterol, and triacylglycerols in humans. Soy isoflavones were considered as the components that contributed to this cholesterol lowering effect. Sirtori

et al. (1997) demonstrated that a marked plasma cholesterol reduction was caused by isoflavone-poor soybean products, suggesting the cholesterol lowering ability of a non-isoflavone component in soy. A few years later the U.S. FDA approved health claims for soy protein and CHD based on its cholesterol lowering effect (Messina *et al.*, 2009). However, the mechanism responsible for the plasma cholesterol lowering ability of soy protein remained questionable until recently. There is evidence suggesting that bile acid binding ability of the peptides that are generated during soy protein digestion contributes to reduce blood cholesterol level (Park *et al.*, 2005).

Bile acids are acidic steroids synthesized in the liver from cholesterol. They are secreted into the duodenum to participate in the digestion process and are actively reabsorbed from the terminal ileum to undergo enterohepatic circulation (Kahlon & Shao, 2004). Studies on the hypocholesterolemic effect of soy proteins have hypothesized that a peptide with high bile acid binding ability could inhibit the reabsorption of bile acids in the ileum and stimulate cholesterol transformation into bile acids in plasma and liver, which will ultimately reduce plasma cholesterol level (Figure 2.5) (Park *et al.*, 2005). Such peptides could also decrease the micellar solubility of cholesterol in the small intestinal epithelial cells and decrease the blood cholesterol level.

Leu-Pro-Tyr-Pro-Arg, which is a fragment of soybean glycinin, has reduced serum cholesterol in mice after oral administration at a dose of 50 mg/kg for 2 days (25.4 % and 30.6 % reduction in total cholesterol LDL-cholesterol, respectively). However, they did not increase the excretion of fecal cholesterol and bile acids indicating that the mechanism responsible for hypocholesterolemic activity might not be the binding of bile acids (Yoshikawa *et al.*, 2000).

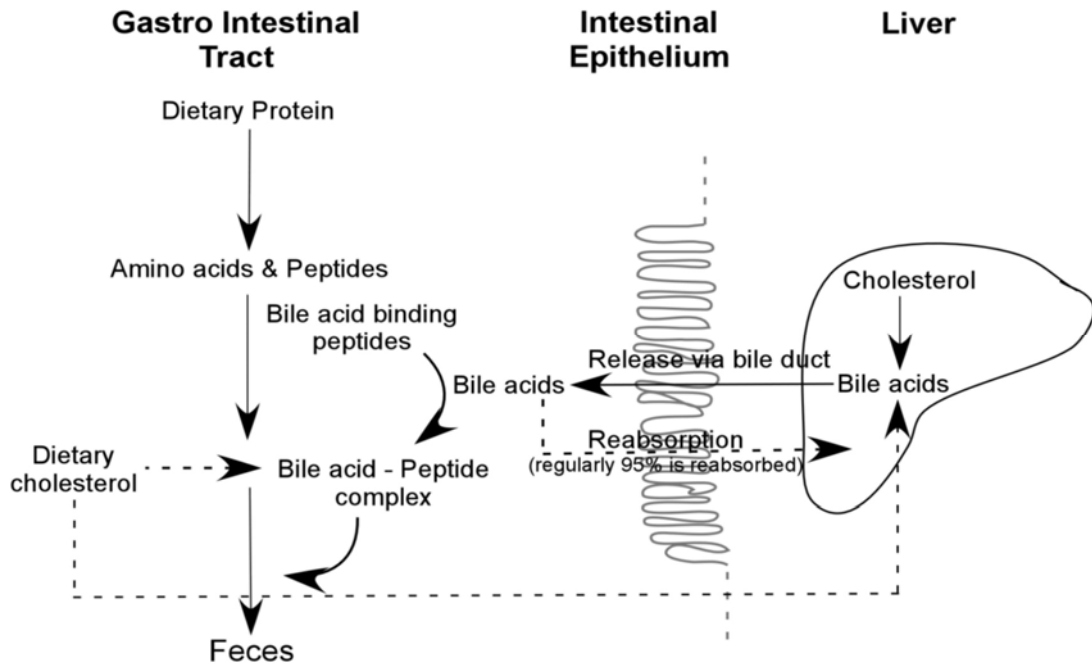


Figure 2.5 Hypocholesterolemic action of bile acid binding peptides. Bile acids are synthesized from cholesterol, conjugated and then excreted into the bile ducts. Released bile acids are reabsorbed via intestinal epithelium facilitating the dietary cholesterol absorption. Dietary protein derived bile acid binding peptides enhance bile acid excretion with feces, reducing the bile acid re-absorption

There are studies on soybean glycinin, which support the hypothesis of bile acid binding action of soybean peptides. It has been demonstrated that soy 11S globulin has higher hypocholesterolemic ability than 7S soy globulin, native soy protein and casein (Park *et al.*, 2005). Of the five subunits (group I: A1aB1b, A1bB2, and A2B1a; group II: A3B4, and A5A4B3) of hexameric soybean glycinin, Choi *et al.* (2002) identified a potential bile acid binding peptide sequence (Val-Ala-Trp-Trp-Met-Tyr) in acidic polypeptide A1a of the A1aB1b subunit. Incorporation of the nucleotide sequence encoding this peptide in the DNA code, which corresponds to amino acids of A1a polypeptide has improved bile acid binding ability of glycinin indicating the ability to improve the physiological functions of SSP by introducing a favorable oligopeptide sequence (Choi *et al.*, 2004). Soy protein peptic hydrolysate with phospholipids (SPHP) has shown significantly greater *in vitro* bile acid binding capacity than that of soy protein peptic hydrolysate without phospholipids (SPH) (Nagaoka *et al.*, 1999). The cholesterol micelles containing SPHP and SPH significantly suppressed cholesterol uptake by Caco-2 cells compared to cholesterol micelles containing casein tryptic hydrolysate. The *in vivo* studies conducted using rats have indicated that the fecal excretion of total steroids was significantly greater in rats fed with SPHP than SPH.

Currently, there are several studies (*in vitro* and *in vivo*) showing the cholesterol lowering effect of different protein hydrolysates (Lovati *et al.*, 2000; Ma & Xiong, 2009; Mendonca *et al.*, 2009; Park *et al.*, 2005; Nagaoka *et al.*, 2001; Yoshie-Stark *et al.*, 2004; Yoshie-Stark *et al.*, 2008) (Table 2.3). Of these, several *in vitro* studies show the ability of food protein derived peptides to bind bile acids, which will lead to cholesterol lowering (Ma & Xiong, 2009; Park *et al.*, 2005; Yoshie-Stark *et al.*, 2004; Yoshie-Stark *et al.*, 2008). In these studies, several proteins such as soy, rapeseed, lupin and buckwheat proteins have also shown bile acid binding in the unhydrolyzed state. However, there is lack of information on characterization (e.g. amino acid sequence) of most of these bioactive cholesterol lowering peptides (Table 2.3).

Table 2.3: Cholesterol lowering mechanisms and identified sequences of food protein derived bioactive peptides

Source Protein	Enzymes	Type of Study	Cholesterol Lowering Mechanism	Amino acid Sequence of Peptide	Reference
Bovine milk β lactoglobulin	Trypsin	<i>In vivo</i> (rats)	Inhibition of micellar solubility of cholesterol which suppresses cholesterol absorption	Ile-Ile-Ala-Glu-Lys	Nagaoka <i>et al.</i> , 2001
Soy protein concentrate	Pepsin & trypsin	Human hepatome cells	Up regulation of LDL receptors	NR	Lovati <i>et al.</i> , 2000
Amaranth protein	-	<i>In vivo</i> (hamsters)	Protein digestibility and excretion of cholesterol and bile acids	NR	Mendonca <i>et al.</i> , 2009
Rapeseed protein	Pepsin & pancreatin	<i>In vitro</i>	Bile acid binding	NR	Yoshie-Stark <i>et al.</i> , 2008
Lupin protein	Pepsin & pancreatin	<i>In vitro</i>	Bile acid binding	NR	Yoshie-Stark <i>et al.</i> , 2004
Buckwheat protein	Pepsin & pancreatin	<i>In vitro</i>	Bile acid binding	NR	Ma & Xiong, 2009
Soy 11S globulin	Pepsin	<i>In vitro</i>	Bile acid binding	Ile-Ala-Val-Pro-Gly-Glu-Val-Ala	Park <i>et al.</i> , 2005

NR: Not reported; LDL: Low density lipoprotein

2.12.4 Antithrombotic activity

The blocking of a coronary artery by thrombi (blood clots) leading to acute arterial thrombosis is the primary cause of most cases of myocardial infarction (heart attack) and about 80 % of strokes. These are collectively the most common cause of death in the developed world (Xiong *et al.*, 2009; Zhang *et al.*, 2008). A key phase of this blood clotting process is the interaction between thrombin and fibrinogen to form the fibrin clot (Zhang *et al.*, 2008). The blood contains fibrinogen and the precursor of thrombin, known as prothrombin. When blood comes in contact with tissues, as in a wound, prothrombin undergoes a series of enzymatic reactions involving Factor VIII, which lead to thrombin as a smaller protein product. The thrombin-catalyzed conversion of fibrinogen to fibrin consists of three reversible steps. Thrombin is involved only in the first step, which is a limited proteolysis to release fibrinopeptides A (FpA) and B (FpB) from fibrinogen to produce a fibrin monomer. These fibrin monomers interact non-covalently to form intermediate polymers (step 2) that aggregate to form the fibrin clot (step 3) (Scheraga, 2004). The clots made upon tissue injury consist of activated platelets and fibrin (Furie & Furie, 1988). The γ chain of human fibrinogen interacts with recognition sites in platelet receptors stimulated by ADP resulting in aggregation. There is a significant molecular similarity between milk clotting (due to action of chymosin on κ -casein) and blood clotting (Erdmann *et al.*, 2008).

Diet, which inhibits platelet activation and aggregation, may reduce the risk of atherothrombosis. Investigations on fruits and vegetables have focused mainly on the biochemical instead of the pathophysiological aspects of atherothrombosis (Yamamoto *et al.*, 2003; Yamamoto *et al.*, 2006; Yamamoto *et al.*, 2008), but most of them were investigated from biochemical aspects, not pathophysiological. In addition, studies on foods of animal origin are rare. However, there are a few studies showing the antithrombotic effect of casein derived peptides (*in vitro* and *in vivo*). The casoplatelins, which are casein-derived peptides, are inhibitors of both the aggregation of ADP-activated platelets and the binding of human fibrinogen to a specific receptor region on the platelet surface. The peptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys derived from bovine κ -casein inhibited platelet aggregation and binding of fibrinogen to

platelets (Jolles *et al.*, 1986). The amino acids Asp, Ile and Lys seem to be important for antithrombotic effect of this peptide as they are homologous in position to the γ chain of human fibrinogen (Erdmann *et al.*, 2008). Therefore, the antithrombotic activity could be the result of competition between the casoplatelin and γ chain of human fibrinogen to bind with platelet receptors. In an *in vitro* and *ex vivo* study using guinea pigs, κ -caseinoglycopeptide (CGP) from sheep, derived from hydrolysis of κ -casein by chymosin, was shown to decrease thrombin- and collagen-induced platelet aggregation. Three peptides obtained from trypsin hydrolysis of CGP (Lys-Asp-Gln-Asp-Lys, Gln-Val-Thr-Ser-Thr-Glu-Val, Thr-Ala-Gln-Val-Thr-Ser-Thr-Glu-Val) were shown to have antithrombotic effects in guinea pigs (Qian *et al.*, 1995). Also, after breast feeding or ingestion of cow's milk-based formulae, antithrombotic CGPs were detected in newborn's plasma, which had been derived from human and bovine κ -caseins, respectively (Chabance *et al.*, 1995). These CGPs are suggested to be released from κ -caseins during the milk digestion process and absorbed into blood circulation. There are a few *in vitro* studies showing the antithrombotic activity of plant derived peptides. Peptides in Alcalase digested rapeseed protein have been shown to inhibit thrombin catalyzed coagulation of fibrinogen (Zhang *et al.*, 2008). Tryptic digests of amaranth glutelin were found to contain antithrombotic peptides (Pro-Pro-Gly, Pro-Gly, and Gly-Pro) that had anticoagulant and fibrinolytic potential of blood plasma (Silva-Sanchez *et al.*, 2008)

2.13 Research evidence on bioactive peptides derived from flaxseed protein

There are reports on the availability of naturally occurring cyclolinopeptides (cyclic, hydrophobic peptides) in flaxseed. These peptides have been identified as Pro-Pro-Phe-Phe-Leu-Ile-Ile-Leu-Val, Pro-Pro-Phe-Phe-Val-Ile-Met-Ile-Leu, Pro-Phe-Phe-Trp-Val-Met-Leu-Met, Pro-Phe-Phe-Trp-Ile-Met-Leu-Met and they are mostly found in the oil fraction (Stefanowicz, 2001). They are reported to be immunosuppressive, hepatoprotective and antimalarial (Stefanowicz, 2001). There are a few recent *in vitro* studies showing the availability of ACEI, renin inhibitory (Udenigwe *et al.*, 2009) and antioxidative (Udenigwe *et al.*, 2009) bioactive peptide sequences within flaxseed protein, which can be released by enzymatic hydrolysis. In these studies, flaxseed

protein was hydrolyzed by proteolytic enzymes such as pepsin, ficin, papain, Thermolysin and pancreatin to release bioactive peptides. Wu *et al.* (2004) reported in a patent that proteolytic digestion of defatted flaxseed meal generates a hydrolysate with ACEI activity. Apart from these studies, there are a few other *in vitro* studies showing calmodulin (CaM) binding activity of flaxseed protein hydrolysates. CaM is an important soluble protein in human platelets that binds with Ca^{2+} and regulates the activity of many cellular enzymes involved in cyclic nucleotide metabolism, Ca^{2+} transport, protein phosphorylation-dephosphorylation cascades, ion transport, cytoskeletal function and cell proliferation (Gnegy, 1993). Omoni and Aluko (2006) reported the ability of flaxseed protein isolate hydrolyzed by Alcalase to yield peptides that can bind to calmodulin. The flaxseed protein hydrolysate inhibited CaM dependent neuronal and endothelial nitric oxide synthase (nNOS). Although nNOS is physiologically important for neurotransmission, vasodilatation, immune response, smooth muscle contraction and relaxation, excessive activity can lead to acute and chronic disorders such as stroke, Alzheimer's, Parkinson's as well as migraine headaches, convulsions and pain (Omoni & Aluko, 2006).

2.14 Information gap on flaxseed protein derived bioactive peptides

The available research evidence shows the presence of bioactive peptide sequences in flaxseed protein that can be released via enzymatic hydrolysis. Of these, especially the ACEI and antioxidant peptides are important to study further as both are related to CVD protection. To the best of my knowledge there is no published information available on the ability of flaxseed protein to generate cardioprotective bioactive peptides during GI digestion. Therefore, it is of interest to investigate whether flaxseed protein possesses bioactive peptide sequences that can be released during GI digestion. Available research data suggest the presence of non-fat and non-lignan constituent in flaxseed that is antiatherogenic. Therefore, it is interesting to investigate whether this constituent is flaxseed protein. None of the published studies have explored the possible amino acid sequence of bioactive flaxseed protein derived peptides. Solid data on bioactivity of products generated upon GI digestion of flaxseed protein obtained by well controlled and well designed *in vitro* studies, *in vivo* animal and human studies will enable the

development of health claims for flaxseed and flaxseed products. Since *in vivo* studies are more expensive to conduct, controlled *in vitro* simulated GI digestion studies on flaxseed protein will be important to generate information on any potential of the protein to release bioactive peptides.

At present flaxseed is consumed as whole seeds, ground or as defatted meal. In whole flaxseed the protein is present with other seed matrix components such as mucilage and oil whereas in the meal the oil is expelled. It is not known which form of flaxseed consumption can provide the health benefits of protein derived bioactive peptides, if any. Such studies will also support bridging the information gap in health benefits of flaxseed constituents. In addition, to improve the demand for flaxseed as a functional food ingredient, there is a need to generate protein products with bioactivities. The flaxseed protein products with bioactive peptides could be used as food ingredients in functional foods or beverages. Since mostly the small peptides will be bioavailable to the human body, there is a need to assess the bioactive nature of peptides released upon hydrolysis of flaxseed protein by commercial microbial enzymes that can cause extensive protein hydrolysis.

3. MATERIALS AND METHODOLOGY

3.1 Materials

Flaxseed of the cultivar Valour was used as the starting material throughout the study. All the enzymes including ACE (from porcine kidney), Flavourzyme[®], pancreatin, and pepsin, and most of the chemicals were purchased from Sigma Chemical Co. Canada. The total bile acid analysis kit and the total dietary fibre analysis kit were purchased from Bio-Quant Diagnostic Kits, U.S.A. and Megazyme International Ireland Ltd., Ireland, respectively. All the chemicals used were of analytical grade.

3.2 Preparation of different flaxseed protein sources

3.2.1 Milled whole flaxseed

The whole flaxseed was milled using a home style coffee grinder and passed through # 35 mesh (500 µm) to obtain milled whole flaxseed.

3.2.2 Milled and demucilaged flaxseed

The mucilage of flaxseed was removed (demucilaging) before milling using water or 0.5 M NaHCO₃ solution as the solubilizing aid. Demucilaging with 0.5 M NaHCO₃ yielded the lowest amount of soluble carbohydrates remaining in the outer seed coat of whole seeds and therefore was used throughout the study for the mucilage removal.

3.2.2.1 Methods of demucilaging

(a) Demucilaging with water

Flaxseeds were soaked in distilled water (1:20, w/v) at room temperature in a beaker overnight with continuous stirring using a magnetic stirrer. The water with extracted mucilage was filtered under suction and discarded. The seeds were rubbed

against a metal mesh using a stainless steel spoon for further removal of mucilage. The seeds were washed with distilled water (1:10, w/v) five times, drained and dried in a forced air oven at 45 °C for 24 h (Alzueta *et al.*, 2002; Wanasundara & Shahidi, 1997).

b) Demucilaging with NaHCO₃

The flaxseeds were stirred in 0.5 M NaHCO₃ (1:8, w/v) in a double jacketed glass vessel at 50 °C for 1 h. The glass vessel was connected to a circulating water bath (VWR, U.S.A.) to maintain a constant temperature. After 1 h, the liquid with mucilage was removed by filtering under suction. Thereafter, the seeds were rubbed against a metal mesh, washed with distilled water, drained and the extraction and washing was continued for two more times. Then the demucilaged seeds were dried as discussed under section 3.2.2.1 (a).

The demucilaged flaxseeds were milled using a coffee grinder and passed through a 500 µm mesh screen to obtain milled and demucilaged flaxseed.

3.2.3 Milled, demucilaged and defatted flaxseed

Two methods of defatting were carried out for demucilaged flaxseed. They were hexane extraction alone and screw pressing using an oil expeller followed by hexane extraction. The method that yielded the lowest residual oil content remaining in the meal was selected and used throughout the study for the removal of oil from demucilaged flaxseeds.

3.2.3.1 Methods of defatting

(a) Hexane extraction

The milled and demucilaged flaxseed was subjected to hexane extraction using n-hexane (milled and demucilaged flaxseed: hexane ratio was 1:3, w/v) for 2 h at room temperature with continuous stirring using a magnetic stirrer. Then the hexane with extracted oil was discarded by filtering under suction and new hexane was introduced. This process was repeated three times (total extraction time: 6 h). The resulting milled,

demucilaged and defatted flaxseed was allowed to dry at room temperature overnight under a fume hood prior to use.

(b) Screw pressing followed by hexane extraction

The demucilaged flaxseeds (before milling) were pressed using an oil expeller (Komet, IBG Monforts Oekotec GmbH & Co., Germany) for partial removal of oil. The resulting meal was milled using a home style coffee grinder, passed through a 500 μ m mesh screen and subjected to hexane extraction using n-hexane as discussed under section (a) of 3.2.3.1.

3.2.4 Isolated form of flaxseed protein

The flaxseed protein was extracted from milled, demucilaged and defatted flaxseed using alkali solubilization followed by acid precipitation. The pH at which flaxseed protein precipitates was determined by investigating the protein solubility profile of the milled, demucilaged and defatted flaxseed.

3.2.4.1 Determination of protein solubility profile at different pH values

This determination was carried out for milled, demucilaged and defatted flaxseed. Fifty mL dispersions of 0.25 % (w/v) sample were prepared at pH values between 1.0 and 10.0. Soluble protein in the sample was extracted with vigorous stirring on a magnetic stirrer at room temperature for 30 min while maintaining the pH using a pH meter (Accumet, Fisher Scientific, U.S.A.) at predetermined values. The solution was then centrifuged using a Beckman J2-HC centrifuge (Beckman Instruments Inc., U.S.A.) at $4220 \times g$ for 15 min. The extract was filtered by vacuum filtration through a #1 Whatman filter paper. The final volume of the filtrate was recorded. The filtrates were frozen at -20 °C until further use. The soluble protein content of the filtrate was determined by the modified Lowry method (Markwell *et al.*, 1978) as described in section 3.3.3. The soluble protein content (%) was plotted against the pH of each flour dispersion to determine the pH, which gives minimum solubility of flaxseed protein.

3.2.4.3 Extraction of proteins

The milled, demucilaged and defatted flaxseed was used for protein extraction. Solubilization of proteins was carried out at pH 8.5 using sample: water of 1:10 w/v and extraction time of 1 h. The pH of the extraction solution was adjusted using 0.1 M and 1 M NaOH. The slurry was then centrifuged at $9820 \times g$ for 20 min to recover solubles and insolubles. After extraction, the residue was subjected to two more extractions (sample: water 1:10 w/v at pH 8.5; time of extraction 1 h) followed by centrifugation similar to above. The supernatants were pooled and the pH was adjusted to 3.8 (the pH that gives minimum soluble protein level). The precipitated proteins were separated by centrifugation at $9820 \times g$ for 20 min and washed once with distilled water. The slurry was centrifuged again as mentioned above. The precipitate was re-dissolved in distilled water and pH was adjusted to 7.0 using 0.1 M NaOH. The protein solution was then dialyzed against distilled water using a dialysis membrane [Spectra/Por, molecular weight cutoff (MWCO): 3.5 kDa] at 4 °C for 48 h to remove any small molecules. During the dialysis period the distilled water used was renewed three times/d. The dialyzed protein solution was then freeze dried. The freeze-dried protein isolate was stored at 4 °C till used for further studies.

3.3 Analysis of composition

3.3.1 Proximate composition

The analysis of moisture (AOAC method 925.09), ash (AOAC method 923.03), crude protein (AOAC method 920.87; $N \times 6.25$) and crude fat (AOAC method 920.85) was carried out using standard AOAC (1990) procedures. The total dietary fibre (TDF), insoluble dietary fibre (IDF) and soluble dietary fibre (SDF) of samples were analyzed using Megazyme total dietary fibre analysis kit and calculated as % values (AOAC method 985.29).

3.3.2 Nitrogen analysis

The total N content of the samples (liquid and solid) were determined according to kjeldhal method (AOAC, 1990; method 920.87) or using combustion N analysis (AOAC, 1997; method 988.05) with a N analyzer (Flash EA[®] 1122, Thermo, U.S.A.)

using EDTA as the standard. The N values obtained were converted to protein content using a conversion factor of 6.25 ($N \% \times 6.25$) when needed.

3.3.3 Modified Lowry method for protein determination

This method was used for liquid samples that were expected to have low levels of protein. If frozen, samples were brought to ambient temperature by immersing in water at same temperature. One milliliter of the sample was mixed with 3 mL of reagent C [prepared by mixing 100 parts of reagent A (2 % Na_2CO_3 , 0.4 % NaOH , 0.16 % sodium tartarate, 1 % SDS, w/v) with 1 part of reagent B (4 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, w/v)] in a test tube. The contents in the tube were incubated for 30 min at room temperature, then mixed with 0.3 mL of Folin-Ciocalteu reagent, vortexed and incubated at room temperature for another 45 min. The absorbance of the resulting solution was measured at 660 nm (Ab_{660}) using a diode array spectrophotometer (HP-8453, Agilent Technologies Canada Inc., Canada). (A standard curve was constructed using bovine serum albumin (BSA; concentration range: 1 $\mu\text{g/mL}$ to 36 $\mu\text{g/mL}$) by plotting its Ab_{660} against different known concentrations of BSA. The soluble protein content of the filtrate was determined by reading the concentration corresponding to the measured Ab_{660} of the sample from the standard curve (Markwell *et al.*, 1978).

3.3.4 Amino acid analysis

The samples were oxidized with performic acid and hydrolyzed with HCl as previously described by Llames and Fontaine (1994) for analysis of amino acids except for Trp. Amino acids were separated using an AMINOSep ion exchange column (particle size: 5 μM ; Capacity: 2 meq/g; Transgenomics Inc., U.S.A.) and post column derivatized with O-phthalaldehyde (OPA) using a HPLC system (Agilent 1100 series, Agilent Technologies, Germany) attached to a fluorescence detector (RF551, Shimadzu, U.S.A.).

For the analysis of Trp, samples (approximately 100 to 200 mg) were weighed in 50 mL Pyrex bottles, and 100 mg of preservative (proprietary formula) was added to each bottle. These preparations were pre-digested in 5 mL of 5 N NaOH for 5 h at room

temperature. Next, the bottles were degassed under vacuum for 5 min, and gently flushed with a constant stream of pure nitrogen for 5 min. Following this, the bottles were loosely capped with screw-cap closures and placed in the oven set at 110 °C. After 1 h of incubation the screw-caps were tightened, and the samples were hydrolyzed at 110 °C for 23 h. The hydrolyzed samples were cooled to room temperature, neutralized to pH between 2.5 to 3.5 with 6 N HCl, and diluted as required. These preparations were centrifuged at $14,000 \times g$, filtered through 0.45 μm syringe filters, and analyzed for Trp content using a HPLC system (Agilent 1100 series, Agilent technologies, Germany). Quantitative analyses were performed using an analytical reversed phase column, (250 x 4.6 mm, 5 μ , Luna, Phenomenex, CA). The mobile phase was 50 mM borate buffer/methanol 70:30 v/v, adjusted to pH 2.8 with 6 N HCl. The mobile phase was delivered at a rate of 1 mL/min and samples were introduced into the column using an auto sampler. Detection was performed using a fluorescence detector set at excitation 233 nm and emission 351 nm. The method was validated for precision, accuracy, reproducibility, and analyte recovery (Olkowski, unpublished).

3.3.5 Determination of soluble carbohydrate content

The soluble carbohydrate content was determined according to the phenol-sulphuric acid method using rhamnose as a standard (Dubois *et al.*, 1956). The water-soluble polysaccharides were extracted from flaxseed by mixing the whole seeds with potassium phosphate buffer (pH 7.0) (1:13 w/v) in a double jacketed glass spinner flask connected to a circulating water bath. The mixture was stirred on a magnetic stirrer for 2 h at 85 °C. The resulting solution was filtered under suction to separate the seeds from the extract. Two mL of the solution was pipetted into a glass test tube and 0.05 mL of 80 % (v/v) phenol was added into the tube. Then 5 mL of concentrated sulfuric acid was transferred into the tube rapidly. The stream of acid was directed against the liquid surface rather than against the side of the test tube in order to obtain a good mixing. The tubes were allowed to stand for 10 min at room temperature. Then they were vortexed and placed in a water bath (Isotemp, Fisher scientific, U.S.A.) at 30 °C for 15 min. The absorbance of the characteristic yellow-orange color was measured at 480 nm against a blank prepared by substituting the polysaccharide extract with distilled water. The

soluble carbohydrate content was determined against a standard curve constructed using rhamnose and was expressed as % of the seed weight.

3.3.6 SDS- PAGE of flaxseed protein

The polypeptide profiles of milled whole flaxseed, milled and demucilaged flaxseed, milled, demucilaged and defatted flaxseed, isolated protein and the residues remaining after simulated gastric and GI digestion/Flavourzyme catalyzed hydrolysis were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing (NR) conditions (Laemmli, 1970). The remaining pellet after centrifugation of the digestion/hydrolysis mix represent the unhydrolyzed protein and partially digested protein during the simulated gastric and GI digestion/ Flavourzyme catalyzed hydrolysis. The N content of the freeze dried undigested residue was determined using a N analyzer prior to SDS-PAGE. The samples (5 mg protein/mL) were dispersed in 0.1 M Tris buffer (pH 8.0) containing 5 % (w/v) SDS in Eppendorf tubes by vortexing vigorously for 2 min. When reducing conditions were required, β -mercaptoethanol (β -ME) was added to the solution to 5 % (v/v) concentration. When NR conditions were required β -ME was not included in the sample buffer. The samples were then placed in an Eppendorf Thermomixer (Brinkmann Instruments Services Inc., Canada) at 99 °C for 10 min with mixing at 1,300 rpm, cooled to room temperature for 10 min and centrifuged (Spectrafuge; Mandel Scientific Inc., Canada) for 10 min at $1,500 \times g$ prior to application. Gradient mini gels (resolving 8-25% T and 2% C, stacking zone 4.5 % T and 3 % C, 43×50×0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4) were used to separate proteins on a PhastSystem equipped with separation and development capabilities (Amersham Pharmacia, Sweden). Sample of ~5 μ g protein was applied into each well and molecular weight standards (Fermentas pre-stained protein ladder ranging from 10 to 170 kDa) were also applied into a separate well. Following separation, the proteins were fixed and stained using PhastGel blue R (Coomassie R-350) and developed to obtain a suitable background color. The gels were scanned and the acquired images were analyzed by the ImageQuant® (version 3.0, Pharmacia Biotech) software.

3.4 Simulated gastrointestinal digestion of flaxseed protein

The GI digestion was simulated *in vitro* using two models; static and dynamic. The static model of digestion was used for milled whole flaxseed, milled and demucilaged flaxseed, milled, demucilaged and defatted flaxseed and isolated protein. The dynamic model was only used for the isolated flaxseed protein. The presence of mucilage, oil and other non-protein constituents in the milled whole, milled and demucilaged and milled, demucilaged and defatted flaxseed products limited the use of the dynamic model for simulated GI digestion. Both models involved a simulated gastric digestion (with pepsin) and a simulated intestinal digestion (with pancreatin and bile) at physiological temperature and pH.

3.4.1 Preliminary studies on simulated gastrointestinal digestion

Two preliminary digestion trials (Static model I and II) were conducted using casein as the standard protein to identify the enzyme to substrate ratios (E/S) that best mimic human GI digestion. The *in vitro* digestion process used in the preliminary trials was the static model of digestion with two phases; gastric phase and intestinal phase. The GI conditions used in the trials were based on published literature. Salivary amylase was not used in any of these trials due to lack of starch in the starting material. The static model I differed from static model II in terms of E/S ratios, volumes of digestive fluids used, pH of the intestinal phase and composition of simulated bile juice. All the other conditions were similar in both models. Details of the preliminary trial using static model I are explained under Appendix I. The methodology used for static model II is explained herein. The most suitable E/S for simulated GI digestion of flaxseed protein was determined based on the digestibility of casein.

3.4.2 Preparation of digestive juices

The simulated digestive fluids were prepared fresh daily as described in the United States Pharmacopeia (USP, 1995) with slight modifications.

3.4.2.1 Simulated gastric control solution and simulated gastric fluid (SGF)

Two grams of NaCl was dissolved in 500 mL of deionized water and the pH was adjusted to 2.0 by adding concentrated HCl. Then water was added to a final volume of 1 L to make the simulated gastric control solution and stored at 4 °C. The cold simulated gastric control solution was mixed with pepsin from porcine mucosa (Sigma, 2490 units/mg solid or 3410 units/mg protein) to a concentration of 4 mg/mL and the resulting solution was used as the SGF.

3.4.2.2 Simulated intestinal control solution and simulated intestinal fluid (SIF)

The intestinal control solution was prepared by mixing 6.8 g of monobasic potassium phosphate in 250 mL of deionized water. Then, 77 mL of 0.2 M NaOH and 500 mL of water was added. The pH of the solution was adjusted to 6.8 by adding 0.2 M HCl/0.2 M NaOH. The resulting solution was then diluted to 1 L with deionized water. The solution was stored at 4 °C. The intestinal control solution was mixed with pancreatin from porcine pancreas (Sigma, 8 x USP) to a concentration of 10 mg/mL to prepare the SIF.

3.4.2.3 Simulated bile solution

The bile solution was prepared by mixing sodium taurocholate and sodium glycodeoxycholate at a concentration of 4 mM each into the simulated intestinal control solution prepared as discussed in section 3.4.2.2. The solution was sonicated at room temperature for 10 min to aid in dissolving bile salts.

3.4.3 Simulated gastrointestinal digestion: Static model

The static model II that does not simulate absorption of small molecules in the intestinal phase was used for digestion.

3.4.3.1 Simulated gastric digestion

The protein (1 g of flaxseed protein or casein) was incubated with gastric control solution (25 mL) in a 50 mL Erlenmeyer flask for 10 min (167 rpm) at 37 °C in a shaking water bath (OLS2000, GRANT, U.S.A.) as shown in Figure 3.1. The solution

was brought to pH 2.0 with 1 M HCl and the total volume was made up to 29 mL with gastric control solution. Freshly prepared SGF (1 mL) was added into this solution to have pepsin: protein ratio (E/S) of 1:250 w/w and the mixture was subsequently incubated in a shaking water bath at 37 °C for 2 h. An enzyme blank was prepared by incubating the enzyme under the described conditions with the substrate omitted. A sample blank was prepared for each sample by incubating the protein under the described conditions without the enzyme. At the end of gastric phase digestion the pepsin activity was inactivated by immediately raising the pH to 6.8 with addition of 1 M NaOH. The solution was then subjected to intestinal phase digestion.

3.4.3.2 Simulated intestinal digestion

The volume of the pH adjusted sample predigested with pepsin was raised to 35 mL by addition of intestinal control solution. The sample was mixed well for 5 min at 37 °C. To this mixture, 1 mL of bile solution and 4 mL of SIF containing pancreatin was added. The trials for digestion of casein with pancreatin were performed using two levels of E/S; 1:/25 and 1/250 w/w. The E/S for digestion of flaxseed protein was selected based on the digestibility of casein. The samples were incubated for 4 h at 37 °C using a shaking water bath. The protocol was also carried out for sample blanks and enzyme blanks as described under gastric phase digestion.

3.4.3.3 Collection of samples

During simulated GI digestion, samples were collected at zero time (unhydrolyzed protein; initiation of digestion), at the end of gastric phase (gastric digest) and at the end of gastric + intestinal phase (gastric + intestinal digest). At the end of each phase the digestive enzymes present in the digested protein samples were inactivated by heating in a water bath at 98 °C for 15 min. The digested samples were then cooled to room temperature and adjusted to the pH at which protein shows minimum solubility (3.8 for flaxseed protein and 4.6 for casein). The samples were then centrifuged at $13,800 \times g$ for 15 min and the supernatant (digest) as well as the pellets (undigested residues) were recovered. The pellets were freeze dried and stored at -20 °C for further analysis. The recovered volume of the digest was recorded and pH was adjusted to 7 using 1 M

NaOH. The resulting solution was adjusted to a known volume using deionized water in a volumetric flask. Aliquots of this solution were stored at -20 °C for the analysis of degree of hydrolysis (DH) and the rest was freeze dried for the determination of bioactivities. The freeze dried gastric and the GI digests were analyzed for ACEI and OH[•] scavenging activities as discussed in sections 3.9.1 and 3.9.2, respectively. All the freeze dried samples were stored at -70 °C until use.

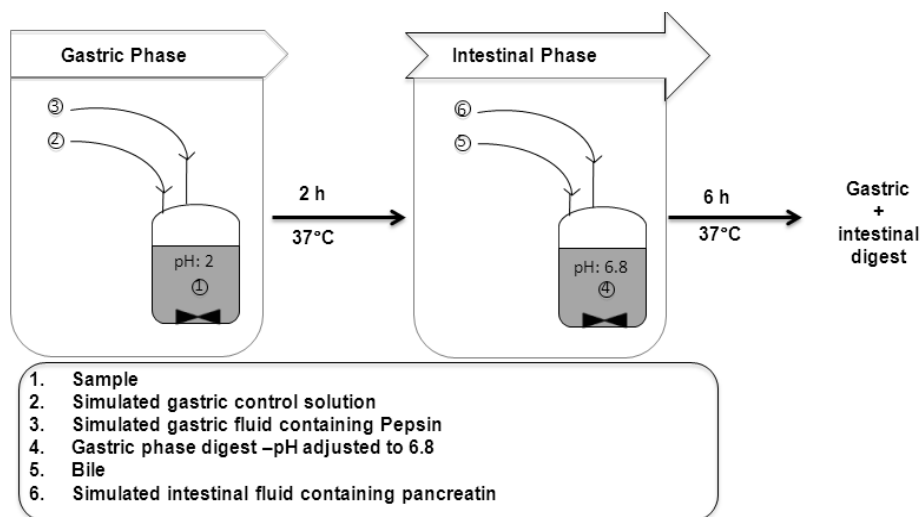


Figure 3.1 Schematic diagram of digestion of flaxseed protein using the static model

3.4.4 Simulated gastrointestinal digestion: Dynamic model

In this model, small intestinal absorption of small peptides was simulated in the intestinal phase.

3.4.4.1 Simulated gastric digestion

The simulated gastric phase used in this method was same as in the static model discussed above.

3.4.4.2 Development of dynamic model for simulated intestinal digestion

In the digestion chamber, a dialysis membrane was used to remove LMW digested products to simulate small intestinal absorption of small peptides and free amino acids.

A preliminary study was conducted to determine the suitability of different dialysis membranes for this study. Two dialysis tubings were tested, namely Spectra/Por® Float-A-Lyzer® (MWCO: 1 kDa, diameter 10 mm, volume: 10 mL) and Spectra/Por® dialysis membrane (MWCO: 1 kDa, flat width 45 mm, diameter: 29 mm, volume: 5 mL) (Spectrum Laboratories Inc., Canada). The ability of these membranes to diffuse digested products was tested using amino acids and peptides of <1 kDa. The amino acid used was Phe (165.19 Da), and the peptides used were glutathione (Glu-Cys-Gly; 307.3 Da) and Gly-Gly-Tyr-Arg (451.5 Da) all prepared separately as 1mg/mL in simulated intestinal control solution. The dialysis tubings with the samples were placed in a double jacketed glass container filled with simulated intestinal control solution (Figure 3.2). The temperature of the system was maintained at 37 °C by connecting the double jacketed container to a circulating water bath set at 37 °C. When Float-A-Lyzers were used, both the contents in the dialysis tube and outer buffer compartment were stirred using a magnetic stirrer. In case of the dialysis membrane, only the outer solution was stirred using a magnetic stirrer. The diffusion of amino acids and peptides from both models were determined by measuring the absorbance of solution inside the membrane at zero time and after 4 h of dialysis. The absorbance was monitored at 280 nm for Phe and at 214 nm for peptides.

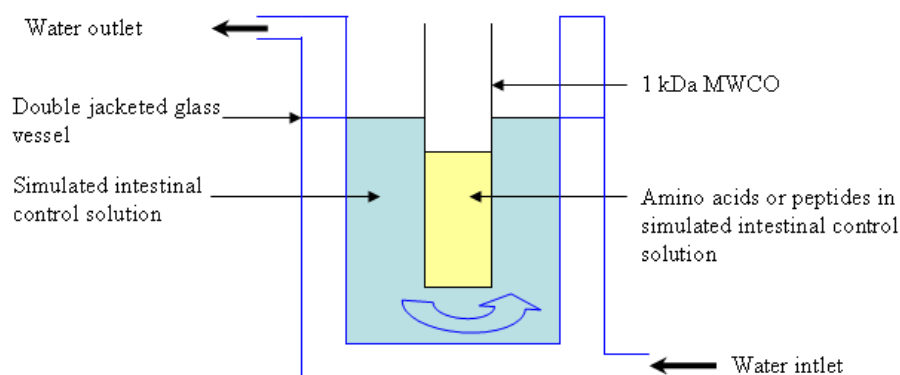


Figure 3.2 Schematic diagram showing the small intestinal digestion in the dynamic model

3.4.4.3 Simulated intestinal digestion

Based on the findings of the preliminary study discussed under the section 3.4.4.2, a dialysis bag made of Spectra/Por[®] dialysis membrane (MWCO: 1 kDa, flat width 45 mm, diameter 29 mm) was used to simulate the intestinal phase of the dynamic model. The dialysis bag was immersed in intestinal control solution (pH 6.8, 1000 mL) in a double jacketed glass vessel which was placed on a magnetic stirrer and connected to a feeding reservoir containing intestinal control solution (maintained at 37 °C) and a receiver flask (Figure 3.3). Feeding fresh intestinal control solution to the vessel containing the dialysis bag and collection of solution containing dialyzed products was done using two peristaltic pumps (variable flow mini pumps, VWR, U.S.A.) providing a constant flow to the system (1.6 mL/min). The temperature of double jacketed container was maintained at 37 °C using a circulating water bath (VWR, U.S.A.).

After the simulated gastric phase digestion the pH of the pepsin digest was adjusted to pH 6.8 and the total volume of the digest was adjusted to 35 mL using intestinal control solution. This digest was transferred to the prepared dialysis bag. One mL of bile salt solution and 4 mL of SIF containing pancreatin previously maintained at 37 °C were added to the digest and further digestion was continued for 4 h with continuous stirring. During the intestinal phase of digestion the dialysate was removed continuously using a peristaltic pump (flow rate: 1.6 mL/min) and collected in the receiving flask. Fresh buffer was pumped into the reaction vessel to replace removed dialysate.

3.4.4.4 Collection of samples

The samples of both dialysate and retentate were collected at the initiation of gastric + intestinal digestion and after 4 h, in addition to the samples of unhydrolyzed protein and gastric digest. The collected samples were heated in a water bath at 98 °C for 15 min to heat inactivate the enzymes. The volume of the collected dialysate was recorded, the pH was adjusted to 7.0 using 1 M NaOH and brought up to a known volume using a volumetric flask by adding water. Aliquots of the dialysate was stored at -20 °C for DH determination and the rest was freeze dried and stored at -70 °C for bioactivity assessment. The retentate (material that was not dialyzed and retained inside the dialysis

bag) was treated similarly to the gastric + intestinal digests collected from static model as discussed above. The recovered pH adjusted supernatant of retentate obtained after centrifugation was used for assessment of DH and bioactivities.

The freeze dried retentate and dialysate of GI digests obtained from the dynamic model were analyzed for ACEI and OH[•] scavenging activities as discussed in sections 3.9.1 and 3.9.2 respectively.

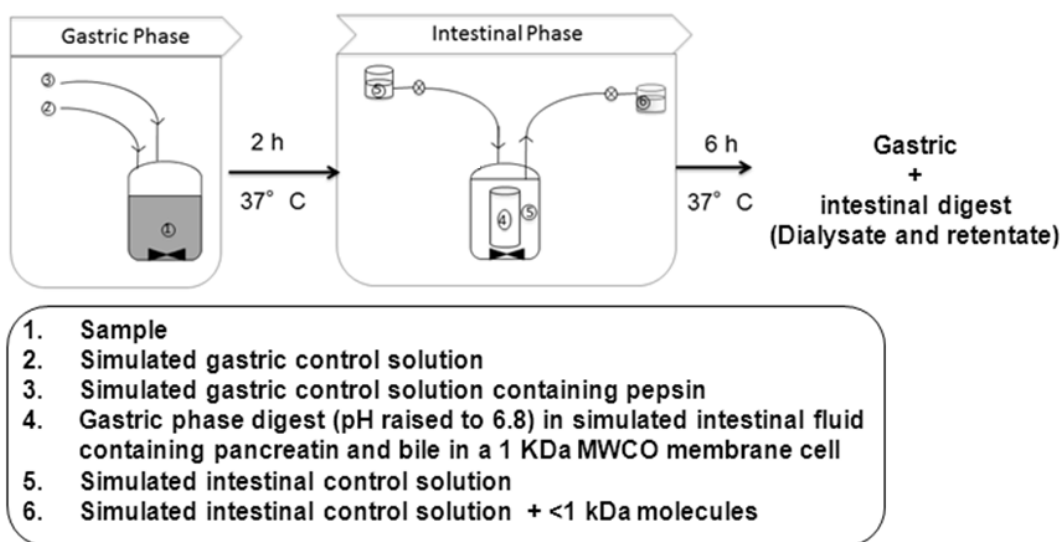


Figure 3.3 Schematic diagram of digestion of flaxseed protein using the dynamic model

3.5 Determination of digestibility

The digestibility of different sources of flaxseed protein was determined as release of TCA soluble N (%) due to pepsin and pancreatin digestion using casein as the standard. A portion of the digest (10 mL) was mixed with equal volumes of 20 % (w/v) TCA. The resulting solution was centrifuged at $13,800 \times g$ for 15 min. The volume of the recovered supernatant was recorded and its pH was adjusted to 7 using 1 M and 0.1 M NaOH. The pH adjusted supernatant was analyzed for N content using a N analyzer using EDTA as the standard (Section 3.3.2). The same procedure was carried out for the enzyme blanks and sample blanks. The % TCA soluble N released during digestion was calculated as follows.

$$\% \text{TCA soluble N} = \frac{(\text{TCA sol N}_{\text{digest}} - \text{TCA sol N}_{\text{sample blank}} - \text{TCA sol N}_{\text{enzyme blank}})}{\text{Total N}_{\text{digest}}} \times 100 \quad (3.1)$$

3.6 Flavourzyme assisted hydrolysis of flaxseed proteins

3.6.1 Experimental design

This experiment was carried out as a two factor central composite rotatable design (CCRD) as described in Kellner *et al.* (1998). The design was used to determine the responses namely DH, and bioactivities of the hydrolysates in relation to the two independent variables, E/S: X_1 and time of hydrolysis (X_2), each at five levels. The variables with the coded and actual levels are given in Table 3.1. The design included nine hydrolysis experiments with different combinations of E/S and time of hydrolysis. Each experiment was carried out in duplicate and the central point of the design (E/S: 47.5 and time of hydrolysis: 12 h/0,0), was repeated four times for reproducibility.

Table 3.1 Experimental design of protein hydrolysis

Variable	Symbol	Levels				
		$-\alpha$	-1	0	+1	$+\alpha$
E/S (LAPU/g of protein)	X_1	1.5	15	47.5	80	93.5
Time of hydrolysis (h)	X_2	0.7	4	12	20	23.3

LAPU: Enzyme activity of Flavourzyme as expressed in Leucine amino peptidase units
 $\alpha=1.414$

3.6.2 Hydrolysis of flaxseed protein

Flaxseed protein was subjected to hydrolysis by the commercially available, food-grade fungal proteolytic enzyme Flavourzyme (Sigma; Activity of 554 LAPU/g) at conditions of its optimum activity (temperature 50 °C; pH 5-7). The combinations of E/S and time of hydrolysis as defined in Table 3.1 were used in the hydrolysis procedure. Each hydrolysis combination was carried out in duplicate in glass double-jacketed spinner flasks, which were placed on magnetic stir plates (Figure 3.4). Prior to protein

hydrolysis, a suspension of isolated flaxseed protein was prepared in deionized water. The pH of the suspension was adjusted to 7.0 by addition of 0.1 M NaOH and thereafter further deionized water was added to maintain the protein: solvent ratio at 1:50 (w/v). The temperature of the hydrolysis mixture was maintained at 50 °C using a circulating water bath (VWR, Canada). At the end of hydrolysis the enzyme was inactivated by heating in a water bath at 95 °C for 10 min. Thereafter the hydrolysate was centrifuged at $8,820 \times g$ for 15 min. Both the supernatant and the unhydrolyzed residue were recovered. The supernatant was freeze dried and stored at $-20\text{ }^{\circ}\text{C}$. The unhydrolyzed residue was stored at $-70\text{ }^{\circ}\text{C}$ for further analysis. In addition to the combination of hydrolysis parameters explained in the experimental design, non-hydrolyzed flaxseed protein as well as untreated flaxseed protein (zero level of Flavourzyme and five levels of time of hydrolysis) were also subjected to hydrolysis experiment as explained above. The freeze-dried crude flaxseed protein hydrolysate (denoted as FPH hereafter) was analyzed for DH and range of bioactivities including % inhibition of ACE, OH^{\bullet} and $\text{O}_2^{\bullet-}$ scavenging activities, ability to inhibit linoleic acid peroxidation, metal chelating activity, antithrombotic activity and bile acid binding ability as discussed in section 3.9.

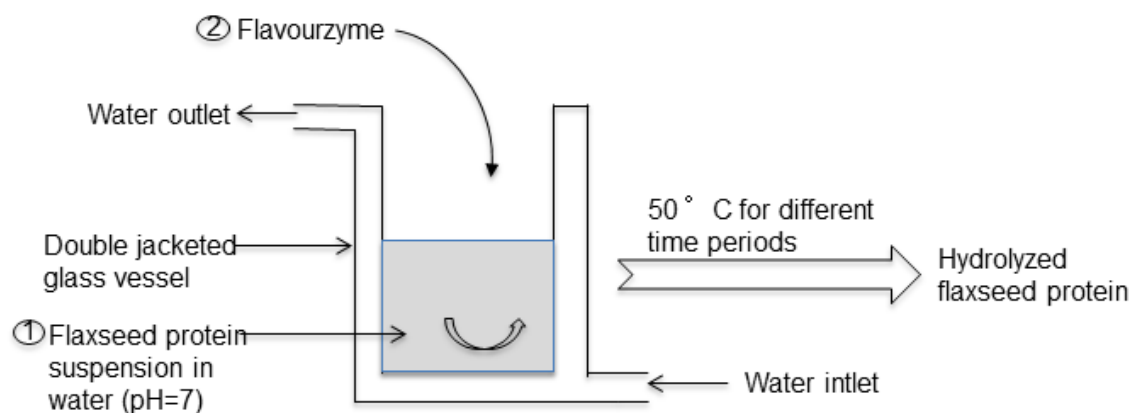


Figure 3.4 Schematic diagram of flaxseed protein hydrolysis with Flavourzyme

3.7 Measurement of degree of hydrolysis and average peptide chain length

The DH, defined as the percentage of peptide bonds cleaved was calculated by analyzing the free amino groups of the reaction mixture according to the OPA method as described by Wanasundara *et al.* (2002), based on a modified procedure of Church *et*

al. (1983). The OPA reagent was prepared daily by mixing 6 mM OPA (first dissolved in 95 % ethanol, v/v) and 5.7 mM DL-dithiothrietol in 0.1 M sodium tetra borate decahydrate containing 2 % w/v SDS. Samples (0.4 mL) were then added to 3 mL of OPA reagent, vortexed , incubated for 2 min at room temperature and the absorbance was monitored at 340 nm using a HP-8453 (Agilent Technologies Canada Inc., Canada) diode array spectrophotometer. The amount of free amino groups was calculated as serine-NH₂ moieties. Total number of amino groups in the flaxseed protein was estimated using acid hydrolysis (6 N HCl at 110 °C for 24 h) assuming complete hydrolysis of all peptide bonds of the protein. The DH and PCL in the hydrolysates were calculated according to the following equations.

$$\% \text{ DH} = \frac{[\text{NH}_2]_{\text{T}_x} - [\text{NH}_2]_{\text{T}_0}}{[\text{NH}_2]_{\text{T}_{\text{Total}}} - [\text{NH}_2]_{\text{T}_0}} \times 100 \quad (3.2)$$

$[\text{NH}_2]_{\text{T}_0}$ = Number of free -NH₂ groups at zero min of hydrolysis.

$[\text{NH}_2]_{\text{T}_x}$ = Number of free -NH₂ groups at the end of enzymatic hydrolysis

$[\text{NH}_2]_{\text{T}_{\text{Total}}}$ = Number of -NH₂ groups liberated by acid hydrolysis (assuming complete protein hydrolysis)

$$\text{Average peptide chain length} = \frac{100}{\text{DH}} \quad (\text{Adler-Nissen, 1986}) \quad (3.3)$$

3.8 Determination of bioactivities

The freeze dried gastric and gastric + intestinal digests of flaxseed protein as well as FPHs were analyzed for bioactivities. The samples were dissolved in respective assay buffers, sonicated for 10 min at room temperature and filtered using 0.45 µm filters (Acrodisc[®] syringe filters, Gelman Laboratory, U.S.A.) prior to determination of bioactivities.

3.8.1 Angiotensin I-converting enzyme inhibitory activity

Angiotensin I-converting enzyme releases hippuric acid from hippuril-L-histidyl-L-leucine (Hip-His-Leu) by hydrolytic removal of the dipeptide, His-Leu (Cushman & Cheung, 1971). Angiotensin I-converting enzyme buffer (ACE buffer; 100 mM Tris HCl, 300 mM NaCl, 10 μ M ZnCl₂ in deionized water, pH: 8.3) was used to solubilize the enzyme, substrate, digest/FPH (2 mg/mL) and the standard (Captopril: ACEI drug). The ACEI activity of the digests/FPHs was assessed with Hip-His-Leu as the substrate. The initial assay mixture consisted of 50 μ L of the substrate (3 mM), 50 μ L of the enzyme (ACE from porcine kidney) solution containing 1.25 milli units of declared enzyme activity and 50 μ L of the digest. The reaction components were separately incubated in glass vials at 37 °C for 30 min using a water bath (Fischer isotope, Fischer Scientific, Canada). Thereafter, the components were mixed and incubated again for 30 min at 37 °C. The reaction was stopped by adding glacial acetic acid (150 μ L). The reaction mixture was then filtered with 0.2 μ m filters (Acrodisc® CR 13 mm syringe filters, Gelman Laboratory, U.S.A.) before subjecting to HPLC. The amount of hippuric acid released due to uninhibited ACE activity was determined as described by Wanasundara *et al.* (2002) with modifications in the mobile phase and detection wavelength. A reversed-phase C18 column (Bond clone C18, 5 μ , 250 \times 4.6 mm) protected by a guard column (Bond clone C18, 10 μ , 50 \times 1.0 mm; Phenomenex, Canada) was used for separation. The isocratic mobile phase, consisted of 25 % (v/v) acetonitrile in deionized water, was adjusted to pH 3.0 by adding glacial acetic acid. The injection volume used was 10 μ L with a flow rate of 1 mL/min. Elution of hippuric acid was detected by monitoring the absorbance at 238 nm. A standard curve was constructed using a series of pure hippuric acid of known concentration in order to quantify the released hippuric acid in the assay mixture. The HPLC system consisted of a solvent delivery system pump (Agilent 1100 series), an auto sampler, LC detector (diode array) and was supported by Chemstation A 10.01 software. The hippuric acid content of a control reaction mixture and a mixture consisting of Captopril (2 mg/mL of ACE buffer) instead of the digests were also determined. The control assay mixture had ACE buffer instead of the hydrolysate. The percent inhibition of enzyme activity was calculated as follows.

$$\% \text{ ACE inhibition} = \frac{[\text{Hippuric acid}]_{\text{Control}} - [\text{Hippuric acid}]_{\text{Sample}}}{[\text{Hippuric acid}]_{\text{Control}}} \times 100 \quad (3.4)$$

3.8.1.1 Determination of inhibition pattern of angiotensin I-converting enzyme

To investigate the inhibition pattern of ACE, the gastric and gastric + intestinal digests/FPHs (0, 0.17 and 0.67 mg/mL) were added to the assay mixture containing different substrate (HHL) concentrations (0.5, 1, 2, 4 and 8 mM). The ACE assay was performed as described above. The Lineweaver-Birk plots were generated to analyze the ACEI pattern of the samples.

3.8.2 Hydroxyl radical scavenging activity

The ability of the gastric and gastric + intestinal digests of flaxseed protein/FPHs to scavenge OH^\bullet was determined using a 2-deoxy D-ribose (DR) assay, originally described by Halliwell *et al.* (1987) with some adaptations as described by Burits & Bucar (2000). The oxidation of 2-Deoxyribose by OH^\bullet formed by the Fenton reaction degrades into malonaldehyde (Gutteridge, 1984; 1987), which can form adducts with 2-thiobarbituric acid (TBA). In this method the ability of digests of flaxseed protein/FPHs to suppress breakdown of 2-deoxy D-ribose (induced via Fenton reaction) into TBA reactive substances (TBARS) was determined. All solutions were prepared fresh. The freeze-dried digests/FPHs were dissolved in KH_2PO_4 -KOH buffer (1.34 mg/mL of buffer, pH 7.4). One mL of the reaction mixture contained 100 μL of 28 mM 2-deoxy D-ribose in KH_2PO_4 -KOH buffer (pH 7.4), 500 μL solution of the digest/FPH, 200 μL of 200 μM FeCl_3 and 1.04 mM EDTA (1:1 v/v), 100 μL H_2O_2 (1.0 mM) and 100 μL ascorbic acid (1.0 mM) in glass tubes. After an incubation period of 1 h at 37 °C in a water bath the samples were mixed with 1.0 mL of TBA (1 % in 50 mM NaOH) and 1.0 mL of 2.8 % TCA. The tubes were then heated at 100 °C for 20 min in a water bath, and cooled to room temperature. The extent of TBARS development was measured by recording the absorbance at 532 nm (Ab_{532}) against a blank using a HP-8453 (Agilent Technologies Canada Inc., Canada) diode array spectrophotometer. Carnosine was used as the standard antioxidant in this assay. A blank sample was prepared by adding all the

reagents except the sample, which was replaced with KH_2PO_4 -KOH buffer and TBA and TCA were added with no incubation at 37 °C.

$$\% \text{ OH}^\bullet \text{ scavenging activity} = \frac{[(\text{Ab}_{532})_{\text{Control}} - (\text{Ab}_{532})_{\text{Sample}}]}{(\text{Ab}_{532})_{\text{Control}}} \times 100 \quad (3.5)$$

3.8.3 Inhibition of linoleic acid oxidation

The thiocyanate assay, with a linoleic acid model system, was used to determine lipid peroxide value of linoleic acid as described by Yen and Hseih (1998). The time taken to form the maximum amount of lipid peroxides was determined using the thiocyanate assay. During the oxidation process peroxides are gradually degraded to form lower molecular compounds. Such degradation products were measured using the TBARS assay. The linoleic acid emulsion was prepared by mixing 560 μL linoleic acid with equal volumes of Tween 20 and 100 mL sodium phosphate buffer (0.1 M, pH: 7.0). The mixture was homogenized using a polytron homogenizer (Kinematica AG, Switzerland) for 10 sec in an ice bath to form the emulsion. A control sample consisting of 1 mL of linoleic acid emulsion, and 1 mL of sodium phosphate buffer was prepared in a screw capped vial in duplicate and incubated in an oven at 40 °C in dark. Aliquots of 0.1 mL were taken from this mixture at 24 h interval during incubation. The peroxide formation was measured according to the thiocyanate method by sequentially adding ethanol (4.7 mL, 75 % v/v), ammonium thiocyanate (0.1 mL; 30 % w/v), control solution (0.1 mL) and ferrous chloride (0.1 mL, 0.02 M in 3.5 % v/v HCl) to the sample. The mixture was allowed to stand precisely for 3 min and the peroxide value was determined by reading the absorbance at 500 nm using a spectrophotometer (Agilent Technologies Canada Inc., Canada). Ascorbic acid was used as the standard. The time of incubation needed for the TBARS assay was determined one day after the absorbance of the control reached maximum value in the thiocyanate method.

For the TBARS assay, the FPH samples were prepared by suspending the freeze dried hydrolysates in sodium phosphate buffer (6.7 mg/mL). Three mL of the emulsion prepared as described for the thiocyanate assay was mixed with 2.4 mL of sodium

phosphate buffer and 600 μ L of hydrolysate sample (final concentration 0.67 mg/mL). The samples were incubated at 40 °C in the dark for 75 h. To 1 mL of sample solution, was added 1 mL of 20 % (w/v) TCA and 1 mL of 0.67 % (w/v) TBA. The mixture was placed in a boiling water bath for 10 min and after cooling was centrifuged at 3000 rpm ($2,060 \times g$) for 20 min. Absorbance (Ab_{532}) of supernatant was measured at 532 nm (Chen *et al.*, 2007). A control sample was prepared by replacing the FPH with buffer. The blank consisted of all the reagents in the control sample to which TCA and TBA were added at zero time of incubation. Ascorbic acid was used as the standard.

$$\% \text{ Inhibition of linoleic acid peroxidation} = \frac{[(Ab_{532})_{\text{Control}} - (Ab_{532})_{\text{Sample}}]}{(Ab_{532})_{\text{Control}}} \times 100 \quad (3.6)$$

3.8.4 Superoxide radical scavenging activity

The $O_2^{\bullet -}$ scavenging ability of FPHs was determined using hypoxanthine-xanthine oxidase (HPX-XOD) system as described by Saint-Cricq de Gaulejac *et al.* (1999). Active oxygen free radicals can be generated by the HPX -XOD system. At pH 7.4, $O_2^{\bullet -}$ reduces the tetrazolium blue into formazan blue (maximum λ_{560}). After addition of radical scavengers, the formation of formazan blue is restricted and absorbance at 560 nm (Ab_{560}) decreases. Absorbance at 560 nm is proportional to the amount of residual $O_2^{\bullet -}$ (Saint-Cricq de Gaulejac *et al.*, 1999).

For the assay, Nitro blue tetrazolium or Tetrazolium blue (NBT; 0.001 M), XOD (1.67 U/mL) and HPX (0.005 M) were solubilized in sodium phosphate buffer (prepared by mixing 4 volumes of 0.1 M Na_2HPO_4 with 1 volume of 0.1 M NaH_2PO_4 ; pH 7.4). The FPHs tested were solubilized in water. The reagent volumes used for blank, control and sample assays were as in Table 3.2. The final concentration of the FPHs in the assay mix was 0.67 mg/mL. The absorbance (Ab_{560}) of control and samples were monitored at 560 nm for 10 min using the kinetic mode of the diode array spectrophotometer (Agilent Technologies Canada Inc. Canada). Standard antioxidants used were carnosine and ascorbic acid at the same concentration as hydrolysates.

Table 3.2 Reagent volumes used for the determination of superoxide radical scavenging activity

	Na phosphate buffer, 0.1 M, pH 7.4	NBT (μL)	HPX (μL)	XOD (μL)	Hydrolysate (μL)
Blank	2400	100	500	-	-
Control	2300	100	500	100	-
Sample	2200	100	500	100	100

NBT: Nitro blue tetrazolium; HPX: Hypoxanthine; XOD: Xanthine oxidase

The $O_2^{\cdot -}$ scavenging activity of the FPHs was determined using the following equation.

$$\% O_2^{\cdot -} \text{ scavenging activity} = \frac{[(\Delta Ab_{560})_{\text{Control}} - (\Delta Ab_{560})_{\text{Sample}}]}{(\Delta Ab_{560})_{\text{Control}}} \times 100 \quad (3.7)$$

$$(\Delta Ab_{560}) = Ab_{560} \text{ at 10 minutes} - Ab_{560} \text{ at zero time} \quad (3.8)$$

3.8.5 Metal chelating activity

The chelation of ferrous ions by the FPHs was estimated by the method of Dinis *et al.* (1994). Divalent iron reacts with ferrozine to form a stable magenta complex, which has a maximum absorbance at 562 nm (Ab_{562}) (Dinis *et al.*, 1994). Reduction of concentration of divalent Fe ions due to chelation was assessed using the reduction of the intensity of the color complex.

In a cuvette 50 μL of FPH in distilled water (final concentration 0.67 mg/mL), 25 μL of 2 mM ferrous chloride and 1375 μL of distilled water were mixed. The mixture in the cuvette was incubated for 5 min at room temperature. Ferrozine (50 μL) was transferred into the cuvette and it was incubated further for 10 min at room temperature. The absorbance of the resulting solution was read at 562 nm (Ab_{562}) against deionized water blank. The control consisted of all the reagents prepared by replacing the FPH with deionized water. The % metal chelation of the FPHs was determined as follows.

$$\% \text{ Metal chelating activity} = \frac{[(Ab_{562})_{\text{Control}} - (Ab_{562})_{\text{Sample}}]}{(Ab_{562})_{\text{Control}}} \times 100 \quad (3.9)$$

3.8.6 Antithrombotic activity

The inhibition of fibrin clot formation by thrombin was measured at 420 nm using a spectrophotometer as described by Szewczuk *et al.* (1992). Bovine fibrinogen (0.1% w/v, 3 mL) in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.1 M NaCl and 0.1 % (v/v) polyethylene glycol 8000 were mixed in a cuvette with FPHs in Tris-HCl buffer at a final concentration of 0.67 mg/mL. The volume of the solution was adjusted with 0.98 mL using Tris-HCl buffer. Human thrombin (20 μ L; final concentration: 0.2 U/mL) was added to the cuvette, mixed and the absorbance at 420 nm (Ab_{420}) was recorded with time. The control reaction consisted of Tris-HCl buffer in place of the hydrolysate. A blank was prepared by including all the reagents present in the control except thrombin. Antithrombin was used as the standard. The antithrombotic activity was determined using the following equation.

$$\% \text{ Antithrombotic activity} = \frac{[(Ab_{420})_{Control} - (Ab_{420})_{Sample}]}{(Ab_{420})_{Control}} \times 100 \quad (3.10)$$

3.8.7 Bile acid binding ability

The *in vitro* bile acid binding capacity of FPHs was assessed as described by Yoshie-Stark and Wasche (2004). In the presence of NAD the enzyme 3- α hydroxyl-steroid dehydrogenase (3- α HSD) converts bile acids to 3-keto steroids and NADH. The NADH formed reacts with NBT to form a formazan dye in the presence of diaphorase enzyme. The dye formation is monitored by measuring the absorbance at 540 nm and is directly proportional to the bile acid concentration in the sample.

The bile acids tested were sodium cholate (SC), sodium glycocholate (SGC), sodium taurocholate (STC), sodium deoxycholate (SDC) and sodium chenodeoxycholate (SCDC). The flaxseed protein isolate and FPHs were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) at a concentration of 6.7 mg/mL, prior to the assay. One hundred μ L of sample suspension was mixed with 900 μ L of 2 mM bile acid solution in sodium phosphate buffer in a micro-centrifuge tube. The mix was incubated at 37 $^{\circ}$ C for 2 h, centrifuged at 16,000 $\times g$ for 5 min using a micro centrifuge (Brinkmann,

Germany). The recovered supernatant was then transferred into a 25 mL volumetric flask. The remaining residue was mixed with 500 μ L of sodium phosphate buffer and centrifuged again. The supernatants were pooled in the volumetric flask and the final volume was adjusted to the mark using sodium phosphate buffer. A control reaction mixture consisting of 100 μ L of sodium phosphate buffer and 900 μ L of 2 mM bile acid solution was incubated along with samples and treated similarly. The blank consisted of all the reagents used for final spectrophotometric analysis except the test sample, which was replaced by the sodium phosphate buffer. Bio-Quant total bile acid analysis kit (Bio-Quant Inc., U.S.A.) was used to quantify free bile acids in the supernatants. All the bile acids were monitored at absorbance 540 nm (Ab_{540}). Calibration curves were constructed for each of the bile acid tested. Cholestyramine resin (0.67 mg/mL), which is the clinically approved bile acid binding agent, was used as the standard. The bile acid binding ability for each bile acid was tested separately.

$$\% \text{ Bile acid binding} = \frac{[(\Delta Ab_{540})_{\text{Control}} - (\Delta Ab_{540})_{\text{Sample}}]}{(\Delta Ab_{540})_{\text{Control}}} \times 100 \quad (3.11)$$

ΔAb_{540} = Change in Ab_{540} after 5 minutes of incubation with reagents in the enzyme assay kit

3.8.8 Determination of IC_{50} value for the bioactivities

The IC_{50} value was defined as the concentration of digests/FPHs (mg/mL) required to achieve 50 % of the control value resulted in for each bioactivity. The IC_{50} values were calculated for each bioactivity tested by using different concentrations of digests possessing the tested bioactivities.

3.9 Purification and characterization of the bioactive peptides

3.9.1 Purification of angiotensin I-converting enzyme inhibitory peptides using angiotensin I-converting enzyme immobilized to glyoxyl-agarose

3.9.1.1 Immobilization of angiotensin I-converting enzyme into glyoxyl-agarose

Angiotensin I-converting enzyme was immobilized, through amino groups of Lys, to 4 % cross-linked (4 BCL) glyoxyl-agarose beads. The immobilized ACE was used to purify ACEI peptides in FPHs as described by Megias *et al.* (2006).

3.9.1.2 Activation of agarose beads

The agarose beads were activated according to Megias *et al.* (2006) before ACE immobilization. Ten mL of agarose was washed using 5 volumes of deionized water and 1 volume of 1 M NaOH containing 0.5 M NaBH₄ to remove the ethanol, which had been used as a preservative in agarose. Five milliliters of 1 M NaOH + 0.5 M NaBH₄ was added to agarose at a bead to solution ratio of 2:1 v/v. While keeping this mixture on an ice bucket, glycidol was added drop wise in order to reach a 2 M final concentration, and the resulting suspension was gently stirred overnight at room temperature. The beaker was kept open while stirring to release hydrogen gas. The agarose beads were washed with 200 mL of deionized water using a scintered bottom funnel under very mild vacuum. Then the agarose beads were suspended in water (100 mL) in a beaker containing NaIO₄ (0.64 g) and stirred for 3 h at room temperature. The oxidation with NaIO₄ converts diol groups into aldehyde groups making multipoint attachment of protein molecules to the gel possible. The resulting activated agarose beads were washed with 300 mL water, mixed with 5 mL of water (10 mL beads in 15 mL suspension) and stored at 4 °C until further use.

3.9.1.3 Immobilization of angiotensin I-converting enzyme to activated agarose

The activated agarose bead suspension (1.5 mL) was centrifuged ($2,285 \times g$ for 30 sec) using a micro centrifuge (Brinkmann, Germany) to remove water. The beads were washed twice with 0.5 mL of 0.2 M NaHCO₃ (pH 10) and transferred into a beaker. It was added with 14 µg of ACE from porcine kidney and 0.2 M NaHCO₃ (pH: 10) (Agarose beads: NaHCO₃, 1:10 v/v). The mixture was stirred gently for 30 min at room

temperature. NaBH_4 (0.011 g) was added to the mixture and stirred for another 30 min. The solution was filtered using a scintered bottom funnel under mild vacuum. The resulting agarose gel with immobilized ACE was washed five times with 10 mL deionized water to remove any unimmobilized ACE. The filtrates were collected, freeze dried and checked for ACE activity (method described in section 3.9.1) to determine the presence of any unimmobilized ACE. The ACE activity of immobilized ACE was determined using unimmobilized ACE as the control. The agarose gel with ACE (approximately 0.8 mL of recovered packed gel) was stored in 0.02 % (w/v) sodium azide at 4 °C.

3.9.1.4 Identification of peptides with angiotensin I-converting enzyme inhibitory activity

The ACE immobilized to agarose beads was used to purify ACEI peptides in the FPH with the highest ACEI activity. The FPH was incubated with the immobilized ACE. Before this, the immobilized ACE was washed with water (700 μL portions) five times and with 700 μL portions of ACE buffer twice to remove any sodium azide present. One hundred μL of ACE immobilized to agarose was mixed with equal volume of ACE buffer. Thereafter 100 μL of this suspension (50 μL packed gel) was pipetted into an Eppendorf tube and was incubated with FPH for 2 h at 37 °C in a water bath. The immobilized ACE: FPH ratio used was 1:2800 (w/w). This ratio was calculated based on the ratio between ACE and hydrolysate used in the ACE assay. After incubation, the supernatant was recovered by centrifuging at $2,285 \times g$ for 30 sec in a micro centrifuge. The agarose beads were washed 4 times with 400 μL portions of ACE buffer to remove any unbound material by mixing with buffer and centrifuged at $2,285 \times g$ for 30 sec. The supernatant and FPH used for incubation was injected to Superdex peptide HR 10/30 column (column size 10×300 mm, Amersham Pharmacia Biotech, Sweden) coupled with AKTA fast protein liquid chromatography (FPLC) system to identify any missing peaks due to binding with ACE. The chromatographic conditions used were same as described under the following section (3.9.2). The immobilized ACE upon incubation with FPH was used for assay of ACE activity as described previously. The immobilized ACE without incubation with FPH was used as the control.

3.9.2 Peptide fractionation using fast protein liquid chromatography (FPLC)

The freeze dried gastric and gastric + intestinal digests of isolated flaxseed protein obtained from both simulated digestion models as well as FPHs that possessed bioactivities were fractionated on a Superdex peptide HR 10/30 size exclusion column (SEC) (column size 10 × 300 mm, Amersham Pharmacia Biotech, Sweden) using an AKTA Explorer FPLC system (Amersham Pharmacia Biotech, Sweden). Of the digests obtained from gastric + intestinal phase of dynamic model, only the dialysate was subjected to fractionation. A solution of the digests/FPHs were prepared in the mobile phase (20 % v/v acetonitrile + 0.05 % v/v trifluoroacetic acid /TFA) to a concentration of 20 mg/mL, and filtered through a 0.45 µm membrane before application. An aliquot of this solution (150 µL) was injected on to the column manually and eluted using isocratic conditions (20 % v/v acetoinitrile + 0.05 % v/v TFA) at a flow rate of 0.5 mL/min at room temperature. The column eluent was monitored at 214 nm and 280 nm. The standards of known molecular masses (insulin chain A, angiotensin II and Hip-His-Leu) were also separated on the same column to determine the distribution of molecular masses of the peptides in the digests. Fractions of eluent (1 mL) were collected using a fraction collector. The collected fractions were pooled based on the molecular mass distribution as determined by comparing with the standards and then freeze dried. The freeze dried fraction of each digest/FPH was assessed for the bioactivities after re-dissolving in respective buffers. The fraction(s) exhibiting the most activity was selected for further purification and characterization of bioactive peptides.

3.9.3 Concentration and purification of peptides

The lyophilized bioactive FPLC fractions of flaxseed protein digests/ FPHs were further purified using PepCleanTM C-18 spin columns (Pierce, U.S.A.) prior to peptide sequencing. The spin column was placed in a receiving tube (Eppendorf tube) and the resin in the spin columns were activated with 200 µL of 50 % v/v acetonitrile, centrifuged at 1500 × g for 1 min and the flow through was discarded. The resin was then equilibrated with 200 µL of 5 % (v/v) acetonitrile having 0.5 % (v/v) TFA, centrifuged at 1500 × g for 1 min and the flow through was discarded. The activation and equilibration steps were carried out twice as instructed by the manufacturer. A

solution was prepared by mixing the sample (1 mg) with sample buffer (100 μ L, 5 % v/v acetonitrile + 0.5 % v/v TFA). This solution was sonicated for 10 min and the solution (100 μ L) was loaded on to the column. The column was centrifuged as before and the flow through was recovered and loaded back to the column to ensure sample binding. The binding process was carried out four times. The resin bound with peptides were then washed with 200 μ L of 5 % (v/v) acetonitrile having 0.5 % (v/v) TFA centrifuged as before and the flow through was discarded. The washing step was repeated four times to remove as much contaminants as possible from the sample. The purified peptides were then eluted into a new receiving tube using 20 μ L of elution buffer (50 % v/v acetonitrile + 0.1 % v/v formic acid). The elution step was carried out four times or until sufficient volume of eluate was collected. The eluate was then freeze dried. The dried samples were suspended in 0.1 % (v/v) formic acid at a concentration of 0.8 mg/mL prior to *de novo* sequencing.

3.9.4 *De novo* sequencing of peptides

The sequence analysis of peptides in the concentrated and purified bioactive fractions of flaxseed protein digests/FPHs was carried out using liquid chromatography (LC) connected to a mass spectrometer (MS). The solvent delivery and sample introduction was performed using Waters Acquity ultra performance LC (UPLC) system (Waters, U.K.). The samples were separated on a 2.1 mm x 50 mm Acquity reverse phase column (BEH, C18, 1.7 μ m) connected to a UPLC using 1 % (v/v) formic acid (solvent A) and acetonitrile (solvent B) as the mobile phases. A gradient elution was employed starting at 10 % (v/v) B and rising linearly to 90 % (v/v) within 15 min. The composition was returned to initial conditions within 2 min and thereafter to 5 % (v/v) B within another 2 min giving a total run time of 19 min. The injection volumes of the sample were 10 μ L. The column was maintained at 65 °C. The standard peptides used were Gly-Gly-Tyr-Arg (451.22 Da) and Phe-Phe (312.15 Da).

Flow from the UPLC column was entered into a high definition mass spectrometer (SYNAPT HDMS, Waters, UK) interfaced with electrospray ionization (ESI) source. The ESI source was operated in the positive ion mode at a capillary voltage of 3.5 V.

Cone voltage and collision energy for peptides were 40 V and 30 eV, respectively. The ion source and desolvation temperature was maintained at 100 °C and 150 °C, respectively. The MS/MS spectra were obtained for the most abundant peptides in the total ion current chromatogram (TIC). The amino acid sequencing of the abundant peptides was performed using Masslynx/MASCOT distiller programs (Micromass, UK) as *de novo* sequencing.

3.10 Statistical analysis

All the experiments and analyses were conducted in duplicate or triplicate. All the values were reported as mean \pm standard deviation. The results of the simulated GI digestion study were evaluated using a generalized linear model (one way analysis of variance-ANOVA) with multiple comparisons of means (Tukey's pair wise comparisons) with probability (*P*) at 0.05.

The data obtained from CCRD experiment of Flavourzyme catalyzed hydrolysis of flaxseed protein was analyzed by ANOVA assuming a general linear model. Response surface methodology and multivariate analysis allowed the determination of the optimum hydrolysis time and E/S combinations that yield the highest level of DH values and bioactivities. Statistical analysis was performed using SAS 9.1 software (SAS Institute Inc., U. S. A.).

4. RESULTS AND DISCUSSION

4.1 Study 1: Release of cardioprotective bioactive peptides during simulated gastrointestinal digestion of flaxseed protein

4.1.1 Materials used for protein extraction

The flaxseed (variety: Valour) used in the present study consisted of 5.74 ± 0.1 % moisture, 4.04 ± 0.0 % ash, 40.62 ± 0.5 % lipid, 22.18 ± 0.4 % protein (N % x 6.25) and 9.84 ± 0.2 % soluble carbohydrates on a fresh weight basis (Table 4.1). The soluble carbohydrate content was mainly analyzed to estimate the mucilage content of the flaxseed. Prior to protein extraction the seeds were subjected to both demucilaging and defatting to remove the mucilage from the seed coat and oil from the seed kernel.

Table 4.1 Proximate composition of flaxseed (cultivar: Valour) used

Component	Value (%) ¹
Lipid	40.62 ± 0.5
Protein	22.18 ± 0.4
Water soluble carbohydrates	9.84 ± 0.2
Moisture	5.74 ± 0.1
Ash	4.04 ± 0.0

¹Values are means \pm standard deviations of triplicate analyses expressed on as is basis

The results of preliminary studies revealed that mild conditions such as soaking in water at room temperature as well as stirring in 0.5 M NaHCO₃ at 50 °C assisted in mucilage removal from the seed coat (Table 4.2).

The flaxseed demucilaged with water as well as with 0.5 M NaHCO₃ had significantly lower ($P<0.05$) soluble carbohydrate content than the whole flaxseed. The use of 0.5 M NaHCO₃ removed a significantly higher ($P<0.05$) level of flaxseed mucilage (88 %) than extraction with water (68 %).

Table 4.2 Estimated soluble carbohydrate levels in flaxseed after treatments

Treatment	Remaining soluble carbohydrate content (%) ¹
Whole flaxseed (no treatment)	12.16 ± 0.6 ^a
Flaxseed, mucilage removed with water	3.84 ± 0.1 ^b
Flaxseed, mucilage removed with NaHCO ₃	2.14 ± 0.1 ^c

¹ Values are means ± standard deviations of triplicate analyses expressed on as is basis (g/100g of seed)

^{abc} Means in the same column followed by the same letter are not significantly different ($P<0.05$)

The isolation of protein from oil-free meals often uses alkali solubilization followed by acid precipitation. This method, suitable for the isolation of protein from soybean, however is not effective for flaxseed because of the polysaccharide gums (mucilage) in the hulls/seed coat (Mazza & Biliaderis, 1989). Flaxseed mucilage is a mixture of polysaccharides present in the epidermal cell layer of the seed coat (Diederichsen *et al.*, 2006), which can be extracted by treating with cold water (Erskine & Jones, 1957). According to Wanasundara and Shahidi (1997), determination of total sugars and pentoses solubilized from the seed coat of flaxseed gives an indirect measurement of its mucilage content. The mucilage of flaxseed seriously interferes with the settling of the aggregated protein due to its swelling and increase in viscosity in the aqueous medium (Smith *et al.*, 1946). Therefore, removal of mucilage from the flaxseed coat was considered as important prior to isolation of proteins. Various methods reported in literature for mucilage removal include soaking seeds with 1 % (v/v) HCl (Mandokohot & Singh, 1979), water followed by passage through a fruit pulper (Madusadhan & Singh, 1983), boiled distilled water (Bhatty, 1993), NaHCO₃ followed by 0.1 N HCl as well as using commercially available carbohydrases (Wanasundara & Shahidi 1997).

Use of NaHCO_3 in the soaking medium improves removal of mucilage as the alkali pH leads to depolymerization of polysaccharides (Wanasundara & Shahidi, 1997).

The mucilage removal of flaxseeds with NaHCO_3 did not require use of specific equipment, acids or high temperature and maintained the protein at its native state. It was also found to be more effective in removing mucilage from flaxseed compared to use of water. Therefore, demucilaging with 0.5 M NaHCO_3 was chosen as the suitable method for mucilage removal throughout the study.

The demucilaged flaxseed was defatted to obtain demucilaged and defatted flaxseed meal. Oil was removed from the demucilaged seeds to improve the recovery of proteins. Of the two methods (hexane extraction of milled whole flaxseed and expeller pressing followed by hexane extraction) used for removing oil from demucilaged flaxseed, it was evident that a product containing <1 % of oil can be obtained using a combination of both methods (Table 4.3). Although hexane extraction and expeller pressing when used alone significantly reduced ($P<0.05$) the oil content in flaxseed, the amount of residual oil remained in the resulting demucilaged and defatted flaxseed was considerably higher compared to that when both methods were combined.

Flaxseed demucilaged with NaHCO_3 and defatted by expeller pressing followed by hexane extraction was selected as the most suitable meal for protein extraction due to low levels of mucilage and oil present (Tables 4.2 and 4.3).

4.1.2 The protein solubility profile of milled, demucilaged and defatted flaxseed against pH

The results shown in Figure 4.1 were used to establish the pH, which provides minimum protein solubility in milled, demucilaged and defatted flaxseed. Solubility of proteins in milled, demucilaged and defatted flaxseed was changed by extraction medium pH as shown in Figure 4.1. The protein solubility curve (Figure 4.1) was a typical 'U' shaped curve similar to most other SSPs, exhibiting a minimum solubility between the pH 3.5 to 4.

Table 4.3 Oil content of whole flaxseed and demucilaged and defatted flaxseed

Seed treatment	Oil content (%) ¹
Whole flaxseed	40.62 ± 0.46 ^a
Demucilaged and defatted flaxseed	
Water demucilaged + Hexane extracted	18.57 ± 0.7 ^c
Water demucilaged + Expeller pressed	23.06 ± 0.3 ^b
Water demucilaged + Expeller pressed + Hexane extracted	0.65 ± 0.0 ^d
NaHCO ₃ demucilaged + Expeller pressed + Hexane extracted	0.23 ± 0.0 ^d

¹Values are means ± standard deviations of triplicate analyses expressed on as it is basis

^{abcd}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

The protein solubility values were greater below and above the pH range of 3.5-4. Therefore, during the extraction of flaxseed protein, the pH of the alkali soluble extract of milled, demucilaged and defatted flaxseed was adjusted in the range of 3.5-4 in order to precipitate maximum amount of proteins. This range is in accordance to the findings of flaxseed protein studies carried out by Painter and Nesbitt (1946), but deviates from findings (pH 4.9-5.8) of Krause *et al.* (2002), Madhusadhan and Singh, (1983) (pH 3-6), Martinez-Flores *et al.* (2006) (pH near 5) and Smith *et al.* (1946) (pH 5.1). It is reported that the solubility of flaxseed nitrogenous compounds are dependent on the composition and ionic strength of the solvent used, solvent/meal ratio and temperature in addition to pH (Dev & Quensel, 1986, Madhusudhan & Singh, 1983). Moreover, the different varieties of flaxseed used in these previous studies also could have an effect on the pH that provides minimum protein solubility.

A protein carries a net positive or net negative charge at pH values below and above its isoelectric pH, respectively. Electrostatic repulsion and hydration of these charged residues promote interaction with water, causing solubilization of the proteins.

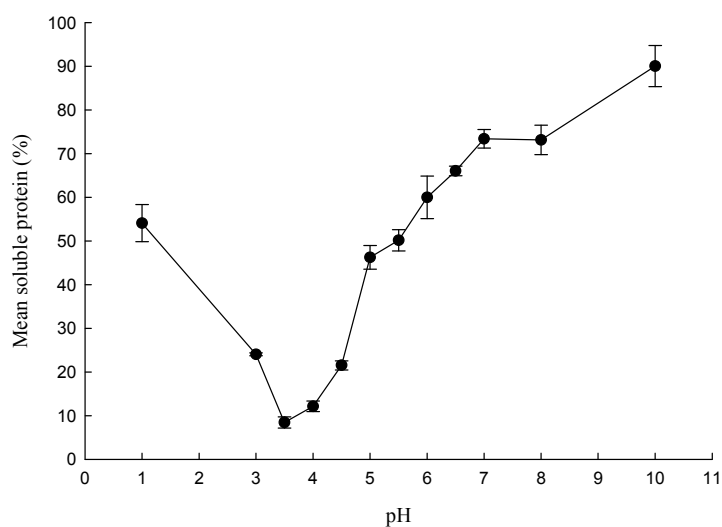


Figure 4.1 Solubility of proteins of milled, demucilaged and defatted flaxseed as changed by medium pH (Mean values of 3 extractions are plotted)

At alkaline pH, the proteins exhibit maximum solubility. When proteins are near their isoelectric pH (IEP), there is an absence of electrostatic repulsion due to charge neutralization in proteins, causing minimum solubility. This condition causes aggregation and precipitation of proteins via hydrophobic interactions (Damodaran, 1996). Therefore, the IEP of flaxseed proteins should lie between the pH of 3.5-4. However, according to the curve (Figure 4.1), approximately 10 % of the proteins in the milled, demucilaged and defatted flaxseed were soluble at the pH range (3.5-4) indicating that all proteins are not exhibiting their charge neutrality at this pH range and likely would not be present in the protein isolate produced.

4.1.3 Protein content of milled, demucilaged and defatted flaxseed and isolated proteins

For the present study, protein was extracted from milled, demucilaged and defatted flaxseed using alkali solubilization followed by precipitation at pH 3.8. It has been reported that several extractions can recover more protein from flaxseed meal than a single extraction (Painter & Nesbitt, 1946). Therefore, two extractions were carried out using water adjusted to pH 8.5 to isolate proteins from milled, demucilaged and defatted

flaxseed. Table 4.4 shows a comparison of the crude protein content of the isolated proteins obtained from different types of milled, demucilaged and defatted flaxseed. The protein content of milled, demucilaged and defatted flaxseed ranged from 30.61 ± 0.2 % (water demucilaging + hexane extraction) to 40.81 ± 0.4 % (NaHCO_3 demucilaging + expeller pressing + hexane extraction). The protein content of isolated flaxseed proteins obtained from different types of milled, demucilaged and defatted flaxseed ranged from 63.48 ± 0.9 to 86.01 ± 0.2 %. The demucilaged and defatted flaxseed obtained from NaHCO_3 demucilaging + expeller pressing + hexane extraction resulted in the protein material with the highest crude protein content (86.01 ± 0.2 %), which was a significantly higher ($P < 0.05$) level than proteins isolated from other demucilaged and defatted flaxseed. These values further revealed that the yield of protein in the isolate increases with the reduction of mucilage and residual oil from the starting material. In a recent report, Udenigwe *et al.* (2009) have reported protein extraction from industrially crushed and defatted flaxseed meal. They have demucilaged flaxseed meal with cellulase enzyme prior to protein extraction using alkali solubilization and acid precipitation. The protein content in the resulting flaxseed protein isolate reported by these authors was 78.9 %, which was lower than the protein content (86.01 ± 0.2 %) of the isolated protein obtained in the present study.

There is a possibility for a certain amount of retained mucilage to be precipitated along with protein during the protein extraction procedure. Presence of a high amount of mucilage (3.31 ± 0.3 %) and oil (23.55 ± 0.2 %) in the protein isolate extracted from water demucilaged and hexane extracted meal can be considered as an important factor which led to lower protein yield. The results of the present study further revealed that the milled, demucilaged and defatted flaxseed prepared by NaHCO_3 assisted demucilaging and expeller pressing combined with hexane extraction was the most suitable material for protein extraction. This term “milled, demucilaged and defatted flaxseed” refers to the above material hereafter. The protein isolated from this material was used throughout the study and will be referred as isolated flaxseed protein.

Table 4.4 Crude protein content of different milled, demucilaged and defatted flaxseed and isolated proteins

Treatment	Protein content (%) ¹	
	Milled, demucilaged and defatted flaxseed	Isolated protein
Water demucilaged + Hexane extracted	30.61 ± 0.2 ^c	63.48 ± 0.9 ^b
Water demucilaged + Expeller pressed + Hexane extracted	38.67 ± 0.5 ^b	68.76 ± 0.3 ^b
NaHCO ₃ demucilaged + Expeller pressed + Hexane extracted	40.81 ± 0.4 ^a	86.01 ± 0.2 ^a

¹Values are means ± standard deviations of triplicate analyses expressed on as is basis

^{abc}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

4.1.4 Composition of isolated flaxseed protein

The proximate composition of flaxseed protein isolated by alkali solubilization followed by acid precipitation is given in Table 4.5. Of the non-protein constituents, the lipid content was found to be relatively low (<1 %), whereas the TDF content was around 8.01 ± 0.6 %. The SDF, which comprised the majority of the TDF was expected to be the soluble polysaccharides (mucilage) from seed coat and cell wall that still remained. Of the dietary fiber in flaxseed, which is concentrated in the hull, two thirds is insoluble fibre consisting of cellulose, hemicellulose and lignin (Tarpila *et al.*, 2005). The results revealed the solubilization and co-precipitation of both soluble and insoluble fibres remained in milled, demucilaged and defatted flaxseed during the protein extraction (See section 4.2.1). The extent of solubilization and co-precipitation with protein was found to be higher in SDF compared to TDF as expected.

The amino acid composition of the milled, demucilaged and defatted flaxseed and isolated protein was also analyzed and is presented in Table 4.6. Both were found to be rich in acidic amino acids such as Glu and Asp as well as the basic amino acid, Arg. The other amino acids found in abundance were the neutral, hydrophobic amino acids, Gly and Leu. Except for Arg, slightly lower levels of all the other amino acids were found in the isolated protein compared to the milled, demucilaged and defatted flaxseed.

Table 4.5 Proximate composition of the isolated flaxseed protein

Constituent	Value (%) ¹
Protein	86.01 ± 0.2
Lipid	0.55 ± 0.1
Total dietary fiber (TDF)	8.01 ± 0.6
Soluble dietary fiber (SDF)	5.41 ± 0.8
Insoluble dietary fiber (IDF)	2.59 ± 1.4
Moisture	5.98 ± 0.9
Ash	4.13 ± 0.3

¹Values are means ± standard deviations of triplicate analyses expressed on as is basis

The most limiting amino acids in the milled, demucilaged and defatted flaxseed were His, Trp, Tyr and the sulphur containing amino acids (Cys and Met), whereas in the isolated protein, Lys and Pro were also limiting in addition to these amino acids. These results were in accordance to the amino acid pattern of flaxseed proteins reported by others (Chung *et al.*, 2005, Oomah & Mazza, 1993). According to Oomah and Mazza (1993) the amino acid pattern of flaxseed protein is comparable to that of soy, with both oilseeds having relatively high levels of Asp, Glu, Leu and Arg. Protein sources rich in Arg and Glu have recently gained popularity due to the preventive function of Arg against heart diseases and Glu in supporting the immune system (Oomah, 2001) and improving athletic performance (Blenford, 1996). L-Arg is the substrate of nitric oxide synthase (NOS) generating nitric oxide (NO) that acts as a vasodilator and an inhibitor of platelet aggregation (Maxwell & Cooke, 1998). Therefore, the low Lys:Arg is suggested to be the causal factor for the antiatherogenic and antithrombotic effects of many plant proteins (Kritchevsky *et al.*, 1981).

Table 4.6 Amino acid composition (g/100 g protein) of milled, demucilaged and defatted flaxseed and isolated protein

Amino acid	Milled, demucilaged and defatted flaxseed ¹	Isolated protein ¹
<u>Essential</u>		
His	2.22 ± 0.0	1.59 ± 0.0
Ile ²	3.91 ± 0.1	3.37 ± 0.0
Leu ²	6.23 ± 0.1	4.67 ± 0.0
Lys	3.56 ± 0.0	2.12 ± 0.0
Met	2.40 ± 0.0	1.69 ± 0.1
Phe	4.97 ± 0.1	4.52 ± 0.1
Thr	4.19 ± 0.1	2.83 ± 0.1
Trp	1.19 ± 0.0	1.10 ± 0.0
Val ²	5.18 ± 0.1	3.75 ± 0.0
<u>Non-essential</u>		
Ala	4.96 ± 0.0	3.77 ± 0.1
Arg ³	9.47 ± 0.4	9.71 ± 0.0
Asp	9.56 ± 0.1	9.02 ± 0.1
Cys	2.75 ± 0.1	2.04 ± 0.0
Glu	21.26 ± 0.2	19.75 ± 0.4
Gly	6.61 ± 0.1	5.19 ± 0.1
Pro	3.27 ± 0.2	2.45 ± 0.1
Ser	5.31 ± 0.1	4.53 ± 0.1
Tyr	2.28 ± 0.1	1.49 ± 0.1
Lys/Arg	0.38	0.22
E/T (%)	43.61	42.28

¹Values are means ± standard deviations of duplicate analyses

² Branched chain amino acids, ³ Conditionally essential (Food and Nutrition Board, 2005)

E/T: Percentage ratio between essential amino acids and total amino acids

The Lys:Arg of the milled, demucilaged and defatted flaxseed and isolated flaxseed protein prepared in the present study was 0.22 and 0.38, respectively. The Lys:Arg of isolated protein from Norman flaxseed is reported as 0.34 (Chung *et al.*, 2005) and is greater than the value of isolated protein obtained in the present study. It was interesting to note that flaxseed protein isolated in the present study had a lower Lys:Arg when compared to that of soy and canola (0.88 for both) (Oomah & Mazza, 2000). The results of the amino acid profile suggest that flaxseed protein has more potential for antiatherogenic and antithrombotic effect than soy or canola protein.

The percentage ratio between essential to total amino acids (E/T %) was also calculated for both milled, demucilaged and defatted flaxseed and isolated protein (Table 4.6). The E/T (%) of isolated protein (42.28 %) and of milled, demucilaged and defatted flaxseed (43.61 %), showed that flaxseed protein in isolation or intact form can contribute significantly to the supply of essential amino acids in the diet. The E/T (%) of flaxseed protein is fairly close to the value (44.4 %) obtained for soybean (Kuri *et al.*, 1991).

4.1.5 Simulated gastrointestinal digestion of flaxseed protein

4.1.5.1 Development of simulated gastrointestinal model (Static model)

In the present study, human GI digestion was simulated *in vitro* using a static model and a dynamic model. The static model simulated major physiological parameters (pH, E/S, temperature, time of digestion) of human GI digestion, except the absorption of digested products via the small intestinal brush border membrane, which was simulated in dynamic model.

In the present study, digestion in the mouth by salivary amylase was not simulated as flaxseed has not been reported to contain starch which is a substrate for salivary amylase. The choice of proteolytic enzymes was considered important because the specific action of enzymes on protein generally influence the composition of digested products. The importance of using crude enzymes corresponding to those found in the gut instead of using purified proteolytic enzymes have been reported for *in vitro* digestion studies (Marable & Sanzone, 1981). Since food proteins occur mainly in close

association with carbohydrates and lipids, it was considered desirable to keep lipases and amylases in the enzymatic preparations. The activity of these nonproteolytic enzymes will influence the proteolytic enzymes, mainly by modifying their substrates (Gauthier *et al.*, 1986). For these reasons, the simulated GI digestion model included pepsin as the gastric phase enzyme and pancreatin as the intestinal phase enzyme. The pancreatin used was a mixture of amylase, trypsin, lipase, ribonuclease and protease (Sigma). Savoie and Charbonneau (1990) have reported the importance of using pancreatin for *in vitro* digestion protocols as the pure enzymes such as trypsin and chymotrypsin are less stable in a mixture.

Another important aspect that was considered in the development of the GI digestion model was selecting a suitable E/S ratio. The E/S ratio is one of the most important variables in digestion studies since the enzymatic release of amino acids and small peptides varies with the ratio (Gauthier *et al.*, 1986). The search of E/S ratio in the published *in vitro* digestion protocols encountered wide range of values. Also humans show a wide variation in their amount and composition of gastric and pancreatic secretions making it more difficult to select an optimal E/S ratio. For example, pepsin in human gastric samples can vary over 10,000 fold (da Silva Gomes *et al.*, 2003) and varies with the type of food consumed (Moreno, 2007). Results of rat studies have shown that dietary proteins can increase the synthesis and secretion of digestive enzymes (both proteolytic and non-proteolytic) and retard the rate of breakdown of these enzymes within the intestine. Products of protein digestion were also found to stimulate pancreatic secretion (Snook & Meyer, 1964). Moreover, many of the published simulated GI studies do not specify the activity of enzymes and the E/S of such studies are estimated on weight basis, which made the comparison between digestion protocols not possible.

Two preliminary studies (Static model I and II, which are described in detail under Appendix I and section 3.4.3 of the Materials and Methodology chapter, respectively) were conducted using casein to identify the most physiologically relevant digestion protocol for protein. Casein is regarded internationally as a standard for protein

digestibility and has been widely studied in *in vitro* systems (Gauthier *et al.*, 1986; Savoie *et al.*, 1988). In the static model I (Appendix I), the human digestive system was simulated considering parameters such as daily protein intake, different volume of digestive juices secreted and their ratios as well as the E/S ratios. All the calculations used in the static model I were based on the human physiological conditions published in the literature (Armand *et al.*, 1995; Okefee *et al.*, 2006). The calculated volumes were scaled down to laboratory scale GI digestion process. In the static model II, the E/S ratios most commonly used by other authors for *in vitro* digestion were investigated. In both studies the digestion was carried out at physiological temperature, mimicking the different pH values and continuous mixing of digest. The major aim of these studies was to identify a suitable E/S ratio for a workable *in vitro* model for flaxseed protein digestion that simulates gastric and intestinal digestion of humans.

The digestibility values obtained for casein from the *in vitro* simulated GI digestion trials (Table 4.7) were compared with the *in vivo* digestibility values available for casein based on human studies (Deglaire *et al.*, 2009). The parameters of the model that contributed to casein digestibility closest to the published *in vivo* values were used to develop the simulated GI digestion model for the study on flaxseed protein digestion.

4.1.5.1.1 Digestibility of casein in static models I and II

The results of both preliminary studies revealed that under the simulated conditions casein digestibility at the end of gastric + intestinal phase increased significantly ($P<0.05$) compared to that of gastric phase (Table 4.7). The static model I showed the lowest digestibility for casein (23.43 ± 2.7 %) at the end of the gastric + intestinal phase. In static model II, the use of pancreatin:casein of 1/25 w/w yielded the highest digestibility for casein (65.46 ± 1.7 %) and it was significantly higher ($P<0.05$) than when pancreatin:casein of 1/250 w/w (37.28 ± 0.9 %) was used. Savoie *et al.* (1988) reported a 97 % digestibility for casein after 24 h proteolysis *in vitro*. Although the digestibility values obtained in the present study were lower than those reported by Savoie *et al.* (1988), the present study considered an intestinal digestion period of 4 h, which could have contributed to the different values.

Table 4.7 *In vitro* digestibility (%) of casein using static digestion models I and II

Digestion phase	Time (h)	Static model I		Static model II	
		E/S (Units/g protein)	<i>In vitro</i> digestibility ^{1,2}	E/S (g enzyme/g protein)	<i>In vitro</i> digestibility ^{1,2}
Initiation of digestion	0	-	No digestion	-	No digestion
Gastric phase	2	12890	13.45 ± 1.9 ^b	1/250	11.71 ± 1.9 ^c
Gastric + intestinal phase	6	130 ³	23.43 ± 2.8 ^a	1/250	37.28 ± 0.9 ^b
				1/25	65.46 ± 1.7 ^a

¹Measured as net % TCA soluble N remaining at the end of each phase

²Values are means ± standard deviations of duplicate analyses

^{abc}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

³USP units/ g protein

A study carried out using human volunteers has shown that digestibility of casein was 64.5 % in human after 140 min (Baglieri *et al.*, 1995). Considering the digestibility values of casein observed at the end of gastric phase as well as gastric + intestinal phase, the E/S in the static model II (pepsin/protein: 1/250 w/w, 10 units/mg of protein; pancreatin/protein: 1/25 w/w, $0.32 \times$ USP units/mg of protein) was selected and used throughout the study as the E/S ratios for flaxseed protein digestion. The static model II will be hereafter referred to as static model throughout the results and discussion section.

These E/S (based on weights) identified in the current study are similar to the enzymatic conditions proposed by Gauthier *et al.* (1986) for *in vitro* digestion of proteins. These authors digested casein in a static gastric phase and a dynamic intestinal phase in a digestion cell, where casein digestion was measured using percent TCA soluble N for gastric phase digestion and percent dialyzed N for intestinal phase. They reported TCA soluble N of 48 % for casein at the end of gastric phase digestion (30 min), and dialyzed N of 27 % after 4 h of pancreatin catalyzed digestion. The digestibility values for casein during gastric phase digestion reported by these authors were greater than that observed in the present study (11.71 % in the selected static model). The specific activities of

pepsin used by Gauthier *et al.* (1986) was slightly lower (3152 units/mg of protein) than that used in the present study (3410 units/mg of protein). The difference in the type of casein used may have contributed for the differences in results. The digestibility value of casein in the simulated intestinal phase of the present study was not comparable with the study of Gauthier *et al.* (1986) due to the differences in the digestion models.

4.1.5.1.2 Amino acid composition of flaxseed protein digests

The amino acid profile of flaxseed protein digests obtained at the end of gastric phase and gastric + intestinal phase using the static model is presented in Table 4.8. There was no pronounced difference between the amino acid profiles of the digests. Similar to the flaxseed protein used (Table 4.8), both digests were rich in Asp, Glu, and Arg. Tyr, a limiting amino acid in isolated flaxseed protein (1.49 %, Table 4.8), was not detected in both digests. This indicates the inability of pepsin or pancreatin to release Tyr containing peptides from flaxseed protein during simulated GI digestion (static model).

4.1.5.1.3 Degree of hydrolysis and average peptide chain length of digested flaxseed protein

Degree of hydrolysis indicates the number of peptide bonds cleaved during the course of GI digestion. Before being subjected to simulated GI digestion, the isolated flaxseed protein in solution showed a low value for DH (3.31 ± 0.0 %) and average peptide chain length (PCL) of 30.2 (Table 4.9).

During digestion with pepsin (gastric phase) and pancreatin (intestinal phase), the DH significantly increased ($P < 0.05$) up to 7.96 ± 0.2 % and 43.95 ± 0.8 %, respectively with further break down of protein giving PCL of 12.5 and 2.3, respectively. Pepsin caused ~5 % increase in DH of flaxseed protein compared to that of undigested. The simulated intestinal phase digestion further increased the DH (~36 %) of the gastric digested flaxseed protein. Results for DH and average PCL suggested that enzymes employed in the simulated intestinal phase contributed more to protein digestion than the gastric phase enzymes.

Table 4.8: Amino acid composition (g/ 100 g protein) of isolated flaxseed protein, gastric and gastric + intestinal digest -Static model

Amino acid	Isolated protein	Gastric digest	Gastric + intestinal digest
<u>Essential</u>			
His	1.59	2.32	2.65
Ile ¹	3.37	4.00	4.22
Leu ¹	4.67	5.74	5.57
Lys	2.12	2.42	2.76
Met	1.69	1.62	1.64
Phe	4.52	5.30	5.34
Thr	2.83	3.68	3.49
Trp	1.10	1.14	1.17
Val ¹	3.75	4.45	5.39
<u>Non-essential</u>			
Ala	3.77	4.53	4.43
Arg ²	9.71	11.36	11.38
Asp	9.02	11.74	11.13
Cys	2.04	2.16	2.42
Glu	19.75	23.78	22.72
Gly	5.19	6.03	6.81
Pro	2.45	2.77	2.28
Ser	4.53	5.66	5.28
Tyr	1.49	0.00	0.00

¹Branched chain amino acids

²Conditionally essential (Food and Nutrition Board, 2005)

Table 4.9 Degree of hydrolysis and average peptide chain length of flaxseed protein and gastrointestinal digests-Static model

Sample	DH (%) ¹	Average PCL
Initiation of digestion	3.31 ± 0.0 ^c	30.2 ^a
Gastric digest	7.96 ± 0.2 ^b	12.6 ^b
Gastric + intestinal digest	43.95 ± 0.8 ^a	2.3 ^c

¹Values

are means ± standard deviations of duplicate analyses

^{abc}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

DH: Degree of hydrolysis

PCL: Peptide chain length

According to Gauthier *et al.* (1986), pepsin hydrolysis facilitates activity of pancreatin on proteins by increasing the solubility and opening the molecular structure, which improves accessibility of peptide bonds. The pancreatin used in the SIF, is a mixture of trypsin, chymotrypsin, elastase, and carboxypeptidase that catalyze collectively to cleave peptide bonds (Ma & Xiong, 2009) causing improved DH. As a result, the peptides with an average of 12 amino acid residues generated during the gastric phase (Table 4.9) were further broken down into small peptides (mainly dipeptides) at the end of intestinal phase digestion (Table 4.9). Even after 4 h of intestinal digestion, DH did not reach 100 %, indicating mostly dipeptides were dominant among the flaxseed protein digestion products, rather than amino acids in free form.

4.1.5.1.4 Bioactivities of the digested flaxseed protein

(a) Angiotensin I-converting enzyme inhibitory activity

The activity of ACE was determined based on the ability of ACE to catalyze the release of hippuric acid from Hip-His-Leu. A reduced amount of hippuric acid was released and detected when ACE was inhibited by the test sample. Figure 4.2 indicates the chromatograms obtained for hippuric acid, which was eluted and detected at a retention time of 5 min under the HPLC conditions used.

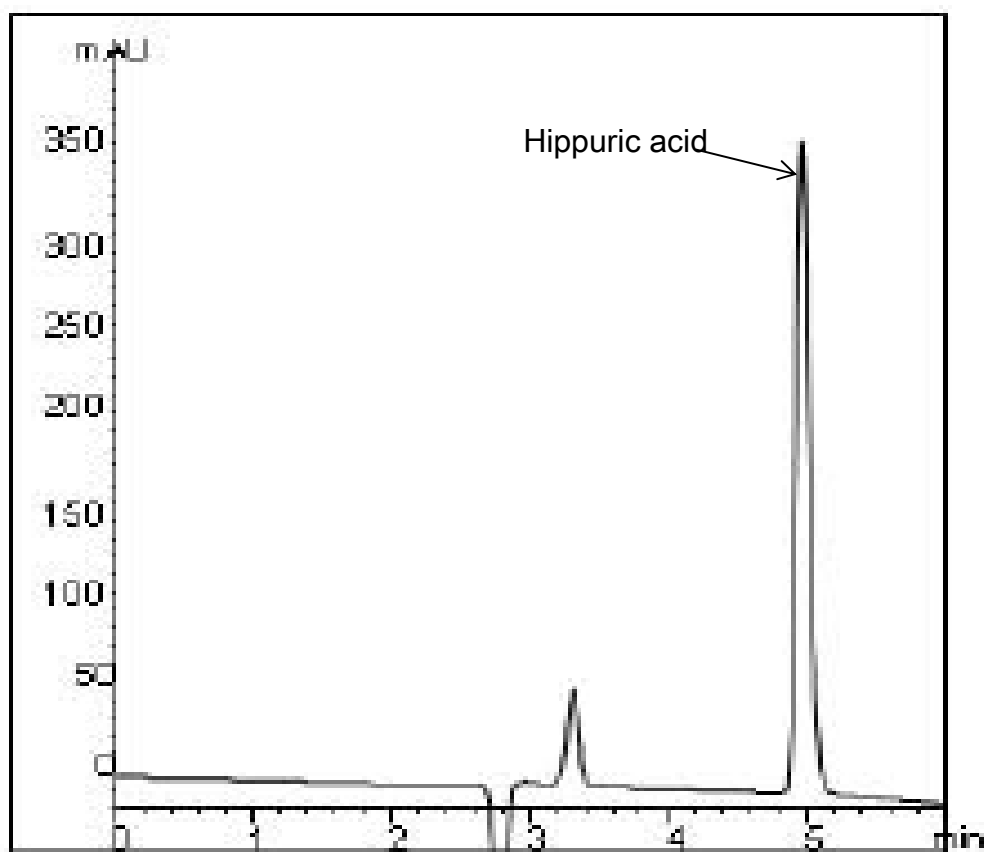


Figure 4.2 HPLC chromatogram for hippuric acid (Concentration: 1 mg/mL, Column: C18, 5 μ , 250 \times 4.6 mm, Flow rate: 1 mL/min, Wave length: 238 nm)

The ACE assay mixture without any digest gave uninhibited release of hippuric acid [Figure 4.3 (a)] whereas those with gastric digest and with gastric + intestinal digest of flaxseed protein showed inhibited release of hippuric acid as shown in Figure 4.3 (b) and (c), respectively. The reduction in released hippuric acid level in the presence of both gastric digest as well as gastric + intestinal digest shows the ACEI activity of these digested protein products. The inhibition by gastric digest was found to be greater than that of the gastric + intestinal digest [Figure 4.3 (b) and (c)].

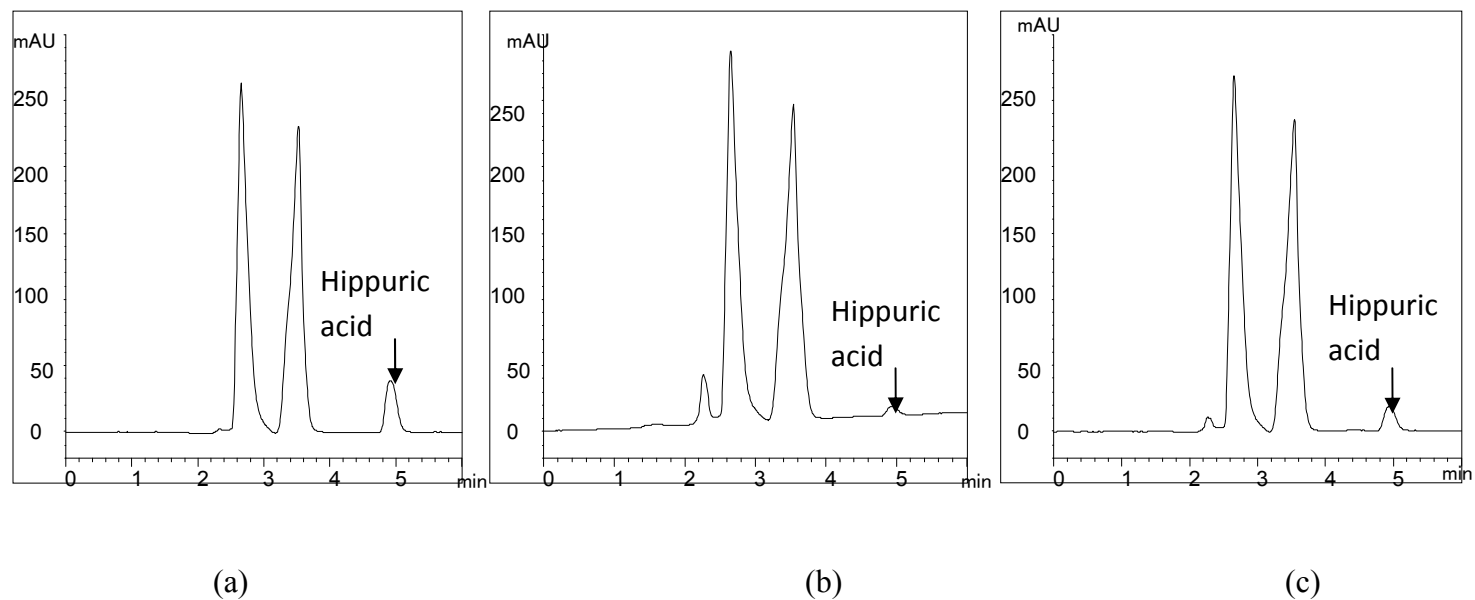


Figure 4.3 HPLC separation of hippuric acid in the assay mix with no ACE inhibitor (a), with gastric digest (b) and with gastric + intestinal digest of flaxseed protein (c) obtained using the static model. HPLC conditions were as follows; Column C18, 5 μ , 250 \times 4.6 mm, Flow rate of 1 mL/min, detection wave length of 238 nm

The undigested flaxseed protein (or sample taken at the initiation of digestion) did not possess any ACEI activity although there was limited hydrolysis (Table 4.10). Interestingly, both gastric and gastric + intestinal digest of flaxseed protein exhibited ACEI activity suggesting that the simulated GI digestion generated peptides with ACEI activity (Table 4.10). This means that the GI digestion released ACEI peptides, which were inactive within the flaxseed protein sequence.

Table 4.10 Angiotensin I-converting enzyme inhibitory activity of flaxseed protein and gastrointestinal digests (static model) in relation to average peptide chain length

Sample	Average PCL	ACEI activity (%) ^{1,2}	IC ₅₀ (mg solids /mL) ¹	IC ₅₀ (mg N/mL) ¹
Initiation of digestion	30.2 ^a	0 ^c	N.D.	N.D.
Gastric digest	12.6 ^b	81.32 ± 1.9 ^a	0.25 ± 0.0 ^b	0.16± 0.0 ^b
Gastric + intestinal digest	2.3 ^c	52.01 ± 1.9 ^b	0.69 ± 0.0 ^a	0.39± 0.0 ^a

¹Values are means ± standard deviations of duplicate analyses

^{abc} Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

²Concentration of the digest was 0.67 mg/mL in the final assay mixture

PCL: Peptide chain length, ACEI: Angiotensin I-converting enzyme inhibitory, N.D: Not determined.

The ACEI activity of flaxseed protein digests generated during different digestion phases were compared. Flaxseed protein derived peptides generated from gastric phase digestion were long chain peptides (PCL: 12.5) and was significantly higher ($P < 0.05$) in the ACEI activity (81.32 ± 1.9 %) than that of the digest of gastric + intestinal phases (52.01 ± 1.9 %), which consisted mainly of short peptides (PCL: 2.3).

Mullally *et al.* (1997) showed that it was the specificity of enzymes and not the final DH that determines the development of ACEI peptides. Several studies have explained the possible reasons for high ACEI activity of peptides generated by pepsin digestion.

The C-terminal residue of ACEI peptides plays a predominant role in competitive binding to the active site of ACE (Cheung *et al.*, 1980). Peptides with hydrophobic and aromatic amino acids as well as the imino acid Pro at the C-terminal are among the most favorable for strong competitive binding to ACE (Clare & Swaisgood, 2000; Cushman *et al.*, 1987; Je *et al.*, 2004). Because pepsin cleaves at the carboxyl end of hydrophobic and aromatic amino acids (Phe, Tyr, Trp, and Leu), it results in peptides with hydrophobic and aromatic amino acids at the C-terminal (Nelson & Cox, 2000). Such peptides bind tightly to ACE at its active site and compete with angiotensin I for occupancy; therefore, ACE cannot bind to angiotensin I and convert it to angiotensin II (Cushman *et al.*, 1987).

The results of the present study showed that further hydrolysis by pancreatin in the intestinal phase reduced the ACEI activity of peptides generated by pepsin assisted hydrolysis (Table 4.10). Similar results were reported for the digests of isolated soy protein (Lo *et al.*, 2006), sunflower protein (Megias *et al.*, 2004) and other food proteins where peptides produced during pepsin digestion had a greater ACEI activity than those produced during pancreatin digestion (Hernandez-Ledesma *et al.*, 2004; Yang *et al.*, 2004). This could be explained by the hypothesis that digestive enzymes in pancreatin split within the sequence of ACEI peptides that were generated by the activity of pepsin on flaxseed proteins. Therefore, the activity of pancreatin caused degradation of pepsin hydrolysis generated ACEI peptides to less inhibitory peptides rather than releasing new peptides with ACEI activity. Moreover, Pripp *et al.* (2004), based on quantitative structure activity relationship (QSAR) modeling, reported that the absence of positive charge at the C-terminal position also increased ACEI potential of milk protein derived peptides. Of the proteases in pancreatic secretions, chymotrypsin cleaves peptide bonds at the carboxyl end of aromatic and hydrophobic amino acids similar to pepsin while pancreatic trypsin preferentially cleaves at the carboxyl end of arginyl and lysyl residues (Nelson & Cox, 2000). Therefore, further cleavage of ACEI peptides in the gastric digest by pancreatic trypsin could have released peptides with positively charged residues at the C-terminal position, causing reduced ACEI activity of gastric + intestinal digest. Maeno *et al.* (1996) have reported an increase in ACE inhibition of casein

derived peptides after pancreatin digestion, which indicates that proteins can behave differently in generating ACEI peptides upon GI digestion.

However, the gastric + intestinal digest of flaxseed protein still showed considerable ACEI activity, which was greater than 50 %. This could be attributed to combined action of proteases in pancreatin. Of the two digests, the ACEI activity of the gastric + intestinal digest is considered more important as it contained a pool of peptides resembling those generated during stomach and small intestinal digestion in the human digestive system. Peptides of the gastric + intestinal digest may be resistant to further hydrolysis by proteases in GI tract and are readily available via small intestine.

The IC_{50} value, the concentration of inhibitory compounds necessary to inhibit 50 % of the activity of ACE, was calculated to further confirm the ACEI potential of the digests. The digest with the lowest IC_{50} possesses highest ACEI activity. The results further showed that the gastric digest exerted the most potent inhibition of ACE with an IC_{50} of 0.25 ± 0.0 mg of solids /mL (0.16 ± 0.0 mg of N/mL) (Table 4.10). The IC_{50} value for gastric + intestinal digest (0.69 ± 0.0 mg of solids /mL; 0.39 ± 0.0 mg of N/mL) was significantly higher ($P < 0.05$) than that of the gastric digest and was higher than that of soy protein digests (0.28 mg/mL) reported by Lo *et al.* (2006). Recent simulated GI digestion studies have reported the IC_{50} for ACEI activity of GI digests of yellow pea protein (0.16 mg/mL; Barbana & Boye, 2010), pea protein isolate (0.07 mg/mL; Vermeirssen *et al.*, 2005) and chick pea (Desi) protein (0.14 mg/mL; Barbana & Boye, 2010). The IC_{50} for flaxseed protein gastric + intestinal digest (0.69 mg of solids/mL) was higher than these values indicating that flaxseed protein digests have low ACEI potency compared to these digests. However, the IC_{50} for flaxseed protein gastric + intestinal digest was within the range (0.35 to 1.73 mg/mL) reported for ACEI activity of GI digested whey protein (Pihlanto-Leppalla *et al.*, 2000). Moreover, ingestion of Thermolysin treated bovine gelatin hydrolysate, which showed an IC_{50} value (0.71 mg/mL) similar to that of flaxseed protein (0.69 mg/mL) upon simulated gastric + intestinal digestion (static model), showed a significant reduction in blood pressure in SHR (Herregodds *et al.*, 2011). Therefore, the ACEI potency of digested flaxseed

protein might be sufficient to cause *in vivo* antihypertensive effects. However, the differences in assay conditions (especially E/S, enzyme activity and time of hydrolysis) as well as units used to express IC_{50} values make it difficult and complicated to compare the values reported in literature with those obtained in the present study.

The ACEI potential of gastric + intestinal digested flaxseed protein was found to be lower than that of Captopril, which showed 100 % ACE inhibition (no hippuric acid released) at the concentration (0.67 mg/mL) used (results not shown). Many studies indicate that ACE inhibitors in protein digests have much higher *in vivo* antihypertensive activity than predicted from *in vitro* assays. This was suggested due to higher affinity of peptides to tissues and slower elimination than synthetic Captopril (Fujita & Yoshikawa, 1999; Marczak *et al.*, 2003). Moreover, under *in vivo* conditions the mode of action of ACEI peptides could be different than that under *in vitro* conditions. Therefore, the ACEI potential of gastric + intestinal digest of flaxseed protein should not be under estimated by comparison with that of Captopril.

The ACEI activity and release of hippuric acid in the presence of flaxseed protein digests in the ACE assay mix was plotted against the concentration of digests and presented in Figure 4.4. The ACEI activity of both digests increased with the increasing concentration of the digest exhibiting a dose dependent relationship.

The ACEI pattern of the gastric and gastric + intestinal digests of flaxseed protein were estimated using Lineweaver-Birk plots (Figure 4.5). The $1/V$ (1/rate of hippuric acid released) by the action of ACE on Hip-His-Leu was plotted against the $1/S$ (1/Hip-His-Leu concentration) in the presence of different levels of digests and the absence of digests. For both digests, the straight lines crossed at the same Y intercept in the presence of different levels of inhibitor, which indicated that both digests possessed a competitive ACE inhibition pattern (Figure 4.5). Therefore, the peptides in the gastric and gastric + intestinal digests could compete with substrate molecules for binding to the active site of ACE enzyme.

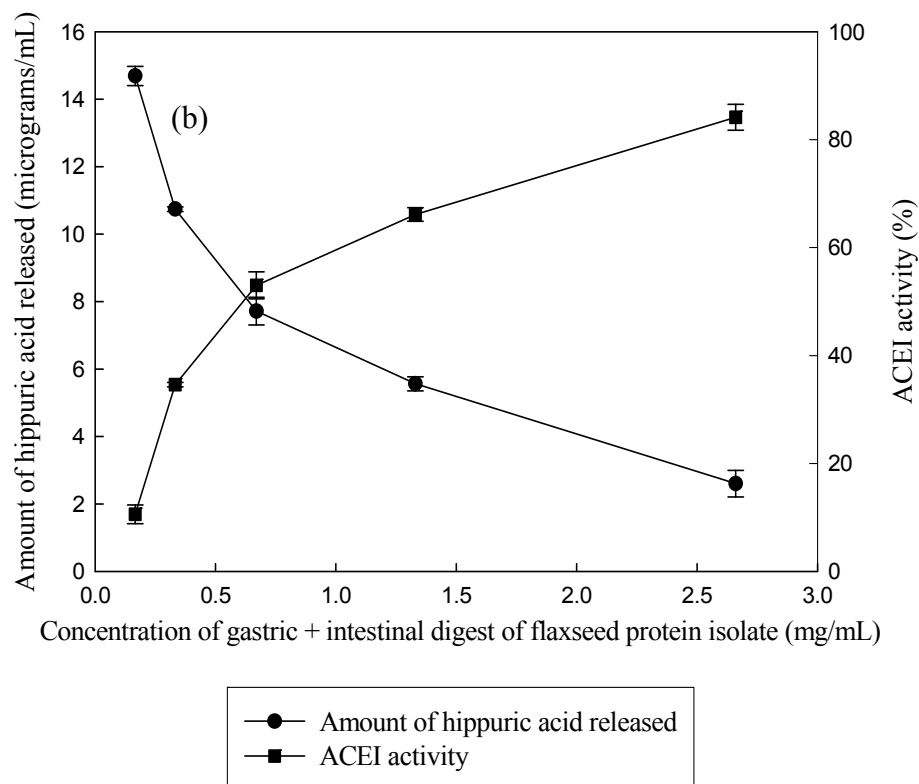
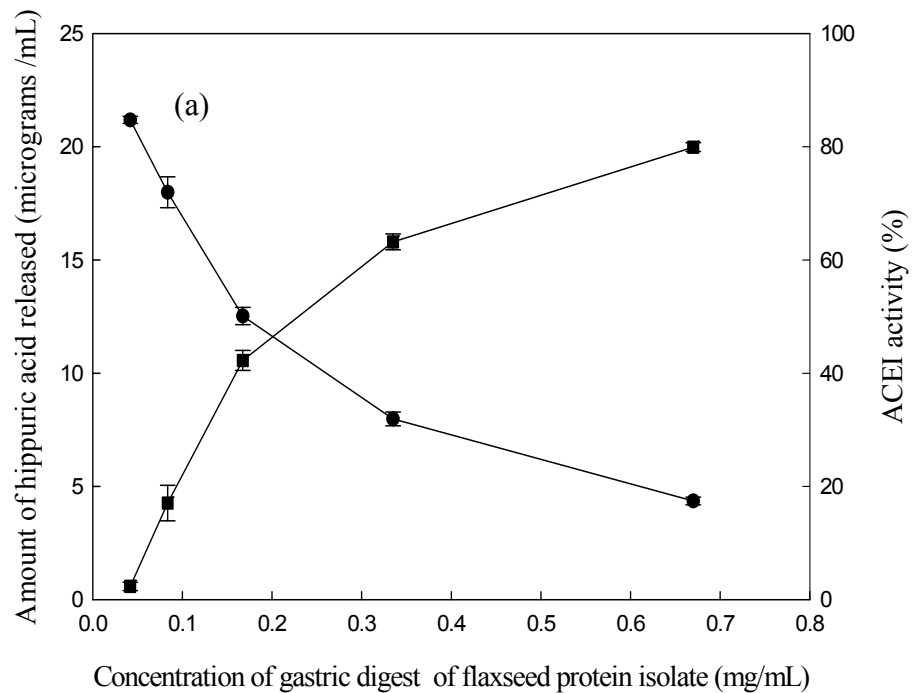


Figure 4.4 Release of hippuric acid and generation of angiotensin I-converting enzyme inhibitory activity by the (a) gastric digest and (b) gastric + intestinal digest in the assay mixture

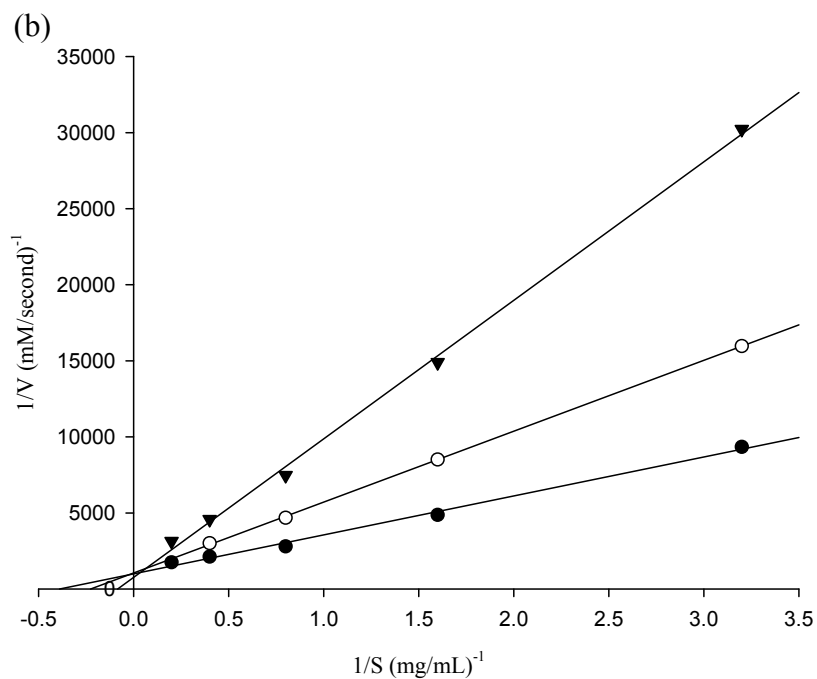
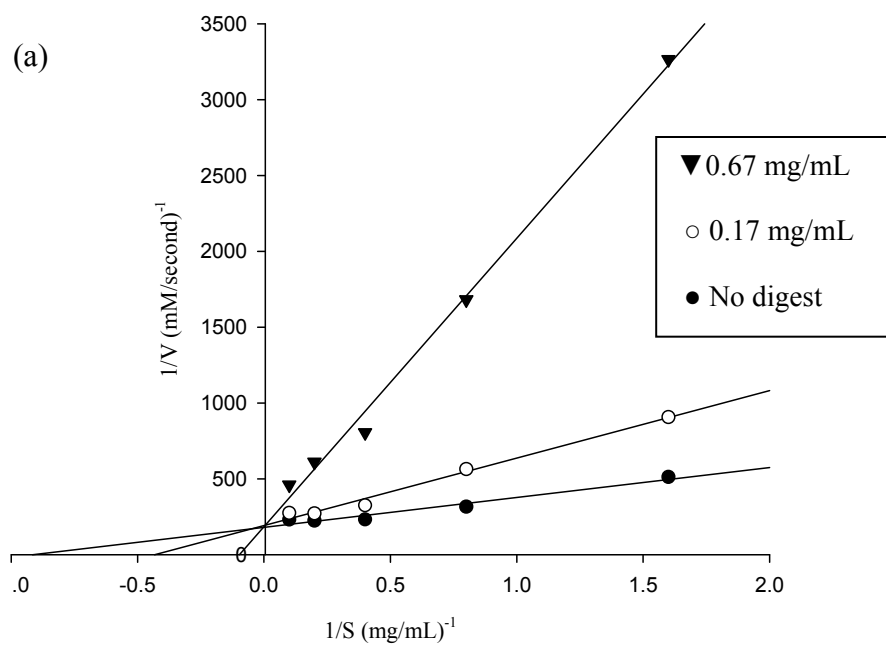


Figure 4.5 The Lineweaver-Burk plots of Hip-His-Leu hydrolysis by angiotensin I-converting enzyme in the presence of different levels of (a) gastric digest and (b) gastric + intestinal digest of flaxseed protein

Most of the food protein derived ACEI peptides are competitive whereas some peptides inhibit ACE activity in non-competitive or uncompetitive mode (Nakagomi *et al.*, 1998).

(b) Angiotensin I-converting enzyme inhibitory activity of flaxseed protein digests under simulated intestinal conditions (No gastric digestion involved) in the static model

An *in vitro* digestion was carried out involving only the small intestinal phase without the gastric phase digestion in order to investigate whether blocking of gastric phase digestion would generate peptides with higher ACEI activity from flaxseed protein than that of gastric + intestinal phase. The resulting digest had a DH (43.71 ± 2.6 %) similar to that of the digest obtained upon simulated GI digestion (Table 4.11). This reveals that small intestinal enzymes alone can break flaxseed protein into small peptides without prior hydrolysis by pepsin. However, the ACEI activity of such digests (46.46 ± 1.7 %) was lower compared to that of flaxseed protein obtained upon simulated GI digestion. This further supports the hypothesis that the mixture of proteolytic enzymes in pancreatin is breaking the ACEI peptides in flaxseed protein within their sequence decreasing the ACEI activity of the released peptides. It also indicates that pepsin digestion prior to small intestinal phase plays a critical role in releasing peptides with ACEI activity from flaxseed protein. When the DH of flaxseed protein increased during GI digestion, the ACEI peptides seems to be further hydrolyzed decreasing the ACEI activity, which was indicated by the increased DH of the GI digested protein.

Recent studies (Marambe *et al.*, 2008; Udenigwe *et al.*, 2009) have reported the ACEI activity of enzymatic hydrolysates from flaxseed protein using food grade proteases. In these two studies, flaxseed protein hydrolysates have been prepared using food grade proteases. To the best of my knowledge, there are no published *in vitro* or *in vivo* studies that report ACEI activity of GI digested flaxseed protein.

Table 4.11 Angiotensin I-converting enzyme inhibitory activity of flaxseed protein under simulated intestinal digestion without gastric phase digestion in the static model

Sample	DH (%) ¹	Average PCL	ACEI activity (%) ^{1,2}
Intestinal digest ³	43.71 ± 2.6	2.3	46.46 ± 1.7

¹Values are mean ± standard deviation of duplicate analyses

²Concentration of the digest was 0.67 mg/mL in the final assay mixture

³Flaxseed protein was subjected to digestion with pancreatin (E/S: 1/25' 37 °C, 4 h) without gastric phase digestion

PCL: Peptide chain length, ACEI: Angiotensin 1-converting enzyme inhibitory

Angiotensin I-converting enzyme plays a key role in regulating blood pressure. Therefore, inhibition of ACE is important in preventing the hypertensive condition, a risk factor for CVD. The results of the present study clearly indicate that flaxseed protein has potential as a food source of antihypertensive agents. So far, ALA and lignans in flaxseed were considered to be the antihypertensive components in flaxseed (Prasad, 2009; Singer *et al.*, 1990). However, there has been no attempt to investigate whether there is any contribution from the protein fraction to the hypotensive effect of flaxseed. The findings of the present study suggest that the antihypertensive agent in flaxseed could be the digested products of proteins. In a recent review, Basch *et al.* (2007) reported that available literature on flaxseed and health includes poor quality studies and do not support recommendation for any health related condition or claims. The authors emphasized the importance of well-designed high quality clinical trials on flaxseed before such recommendations. The present study provides useful information on antihypertensive potential of flaxseed protein at *in vitro* level, which warrants appropriate *in vivo* studies.

(c) Hydroxyl radical scavenging activity

The OH[•] scavenging activity of flaxseed protein digests were determined using an assay containing OH[•] generated by the Fenton reaction between Fe³⁺ and H₂O₂ similar to the physiological conditions. The OH[•] scavenging activity of GI digests is shown in Table

4.12. Similar to ACEI activity, the undigested protein did not show any OH[•] scavenging activity. However, in contrast to the ACEI activity, OH[•] scavenging activity was not present in the digest at the end of simulated gastric digestion of flaxseed protein (Table 4.12). The recent study by Udenigwe *et al.* (2009) also reported similar OH[•] scavenging activities of <1 kDa fraction of pepsin hydrolyzed flaxseed protein. In the present study, 4 h digestion of simulated intestinal phase with pancreatin caused a rise in OH[•] scavenging activity to 45.56 ± 1.1 % with an IC₅₀ value of 0.70 ± 0.1 mg of solids/mL (0.40 ± 0.0 mg of N/mL) (Table 4.12).

Table 4.12 Hydroxyl radical scavenging activity of digested flaxseed protein in relation to degree of hydrolysis and average peptide chain length

Sample	DH(%) ¹	Average PCL	OH [•] scavenging activity (%) ^{1,2}	IC ₅₀ (mg of solids /mL) ¹	IC ₅₀ (mg of N /mL) ¹
Initiation of digestion	3.31 ± 0.0^c	30.2	0 ^b	N.D.	N.D.
Gastric digest	7.96 ± 0.2^b	12.6	0 ^b	N.D.	N.D.
Gastric + intestinal digest	43.95 ± 0.8^a	2.3	45.56 ± 1.1^a	0.70 ± 0.1	0.40 ± 0.0

¹Values are mean \pm standard deviation of duplicate analyses

^{abc}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

²Concentration of the digest was 0.67 mg/mL in the final assay mixture

DH: Degree of hydrolysis, PCL: Peptide chain length, OH[•]: Hydroxyl radical

N.D.: Not determined

You *et al.* (2009) have reported similar increase in OH[•] scavenging activity when gastric digestion was followed by intestinal digestion. The digest generated in the simulated gastric + intestinal phase exhibited an increase in OH[•] scavenging activity with an increase in concentration (Figure 4.6). The results revealed that the small peptides (average PCL: 2.3) generated by the pancreatin digestion of flaxseed protein have potential to scavenge OH[•] whereas no OH[•] scavenging potency was displayed by the long chain peptides (PCL:12) generated by pepsin. Udenigwe *et al.* (2009) digested flaxseed protein with pancreatin and reported that <1 kDa ultrafiltered fraction of the

resulting hydrolysate had a OH^\bullet scavenging activity with an IC_{50} of 0.06 mg protein/mL. However, their study was not conducted under simulated GI digestion conditions and therefore flaxseed protein had been hydrolyzed without a pre-digestion step with pepsin, making it difficult to compare the values for OH^\bullet scavenging activity with the findings of the present study.

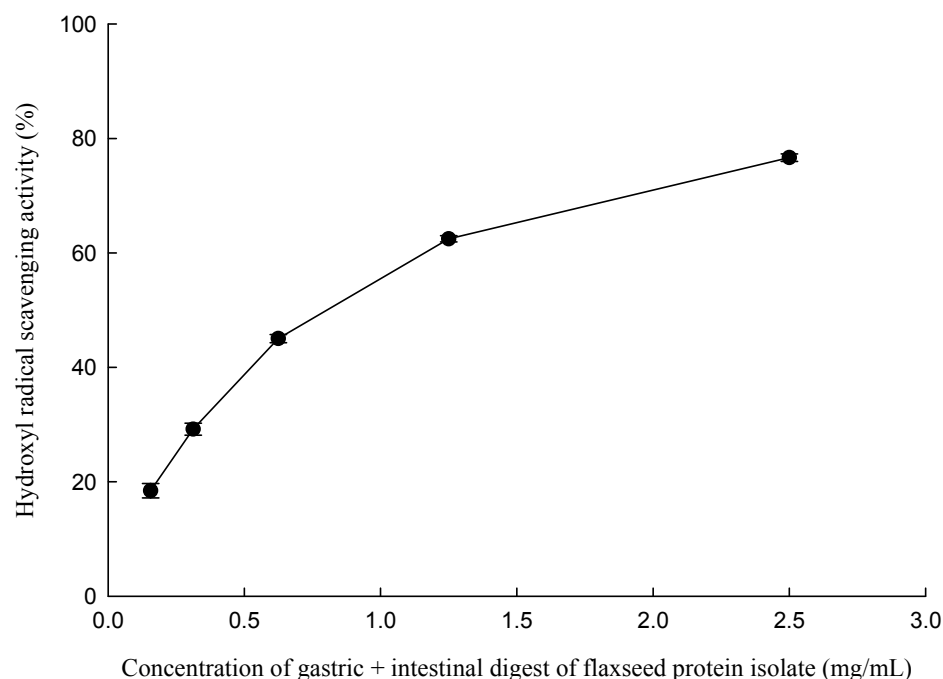


Figure 4.6 Hydroxyl radical scavenging activity of gastric + intestinal digest of flaxseed protein in relation to the concentration of digest in the assay mixture

Carnosine (0.67 mg/mL), the standard antioxidant used, had a OH^\bullet scavenging activity of 63.50 % (results not shown) that was greater than that of the gastric + intestinal digest of flaxseed protein ($45.56 \pm 1.1\%$). According to Chen *et al.* (1996; 1998), the antioxidant activity of protein hydrolysates is related to the amino acid composition and sequence, the size and configuration of the peptides present. The presence of amino acid residues such as His, Lys, Tyr, Met and Pro were significantly correlated with radical scavenging activity of peptides (Pena Ramos *et al.*, 2004; Saito *et al.*, 2003; Tsuge *et al.*, 1991). Tripeptides with Trp or Tyr at their C-terminus exhibited very strong radical-scavenging activity (Saito *et al.*, 2003). Since radical-scavenging is likely due to the

hydrogen donor activity of the hydroxyl groups of aromatic amino acids, the presence or absence of such amino acids in the peptide would affect its radical scavenging capacity. Based on a study on porcine myofibrillar protein, Saiga *et al.* (2003) reported that short peptides rich in acidic amino acid residues (Asp, Glu) exhibited strong antioxidant activity. There is also evidence showing that the antioxidant activity of certain amino acids is enhanced when incorporated into dipeptides (Erdmann *et al.*, 2008), suggesting the crucial role of the peptide bond and/or specific structural features of the peptides regarding antioxidant potential. Contradictory results have been reported by Hernandez-Ledesma *et al.* (2005). Based on results of a study on antioxidant peptides in hydrolyzed whey protein, these authors suggested that peptide bond or structural confirmation of peptides reduced the antioxidant activity of constituent amino acids. It was also noted that when in a peptide, antagonistic effect between amino acids would reduce their own radical scavenging activity (Hernandez-Ledesma *et al.*, 2005).

The aromatic amino acid content of the gastric and gastric + intestinal digest of flaxseed protein showed no pronounced differences (Table 4.8). The aromatic amino acid Tyr was absent in both digests but present in isolated protein. However, both gastric and gastric + intestinal digests of flaxseed protein were rich in Asp and Glu (Table 4.8). The significant improvement in OH[•] scavenging activity observed for the gastric + intestinal digest of flaxseed protein compared to the gastric digest may be attributed to the short PCL (dipeptides) in the gastric + intestinal digest or the difference in amino acid composition in the peptides caused by cleavage specificity of different digestive enzymes.

Previous studies have reported the antioxidant potency of flaxseed non-protein components, in which SDG is thought to be the responsible component. Prasad (1997) showed that isolated SDG has OH[•] scavenging *in vitro*. However, human studies conducted by Hallund *et al.* (2006) suggested that lignan may not be the antioxidant component in flaxseed. In the present study we did not analyze the SDG level of isolated flaxseed protein. However, the absence of OH[•] scavenging activity in the digest collected at the initiation of digestion of flaxseed protein (Table 4.11) and sample

blanks (results not shown) suggested that there was no contribution of SDG to the antioxidant activity of flaxseed protein digests and the observed OH[•] scavenging activity was solely due to the products generated during GI digestion of flaxseed protein. Recent *in vitro* studies conducted by Marambe *et al.* (2008) as well as Udenigwe and Aluko (2010) using fungal proteases and Udenigwe *et al.* (2009) using individual proteolytic enzymes also confirmed the presence of antioxidant peptide sequences in flaxseed protein, which can be released upon hydrolysis (These results are further discussed in the section 4.3.2.2.1 in this thesis).

The OH[•] are the strongest radical scavengers among ROS, which easily react with any biomolecule. In the myocardial cell membrane, OH[•] can initiate lipid peroxidation or oxidation of protein sulfhydryl groups. Lipid peroxidation leads to fragility of lysosome membrane and causes leakage of hydrolytic enzymes. This leads to cell damage and death leading to cardiovascular injury (Horwitz & Rosenthal, 1999). Therefore, the results suggest that upon human consumption flaxseed protein may act as a source of antioxidant peptides that will fight against OH[•] formation thereby preventing CVD.

4.1.5.2 Digestion of flaxseed protein in the dynamic model

The dynamic model of GI digestion contained gastric and intestinal phases in separate reaction chambers. The gastric phase of digestion in this model was similar to that of the static model. The intestinal phase differed from the static model due to continuous removal of digested products that simulated absorption in the small intestine.

Preliminary experiments were carried out using a Float-A-Lyzer® (ready to use cylindrical dialysis tubing made of cellulose ester) and a dialysis bag for the determination of their suitability as the small intestinal compartment in the dynamic model and the results are as in Table 4.13. Both the Float-A Lyzer and the dialysis bag were tested for their ability to diffuse amino acids and LMW peptides that mimic products of GI digestion as described in section 3.4.4.2 (Materials and Methodology chapter of this thesis). Although both were made of dialysis membranes of 1 kDa MWCO, the Float-A-Lyzer was found to be less efficient when compared to the

dialysis bag with respect to diffusion of molecules within 4 h (the time used for intestinal phase digestion). Among the molecules tested, Phe was the only molecule that was able to diffuse out of the Float-A-Lyzer during the 4 h period, whereas Phe and the peptides (glutathione and Gly-Gly-Tyr-Arg) were able to diffuse out of the dialysis bag as indicated by the reduction of optical density (O.D.) of substances in the membrane (Table 4.13). Therefore, the dialysis bag (MWCO: 1 kDa) was used for the further studies on simulated GI digestion using the dynamic model.

Table 4.13 Diffusibility of selected amino acids and peptides through the Float-A-Lyzer and dialysis bag

Amino acid/peptide	Optical density			
	Float-A-Lyzer		Dialysis bag	
	<u>Initial</u>	<u>After 4 h</u>	<u>Initial</u>	<u>After 4 h</u>
L-Phe (MW: 165.19) ¹	0.192	0.071	0.278	0.05
Gly-Gly-Tyr-Arg (MW: 451.5) ²	3.0	3.0	3.0	2.7
Glutathione (MW: 307.3) ²	3.0	3.0	3.0	0.128

¹Optical density was measured at 280 nm

²Optical density was measured at 214 nm

MW: Molecular weight

Use of dialysis membrane with a MWCO less than 5 kDa would be the reasonable approach for mimicking *in vivo* absorption, considering the pore diameter and the permeability characteristics of the human intestine (Ekmekcioglu, 2002). In the development of a digestion cell for determining protein digestibility, Gauthier *et al.* (1982) chose a dialysis membrane with a MWCO of 1 kDa. The end product of protein digestion in the gut is a mixture of amino acids and short peptides of 2-6 amino acids (Peters, 1970). The dialysis membrane with 1 kDa MWCO selects nitrogenous material ranging from free amino acids to peptides possibly 8-10 amino acids long, which is very close to the end products of intestinal digestion (Gauthier *et al.*, 1982). Therefore, in the present study the dialysis bag of MWCO 1 kDa was used to simulate the intestinal compartment and was expected to closely mimic absorption *in vivo*.

Filter grown Caco-2 cells or dialysis membranes are usually used as tools in experimental models for studying the transport of nutrients between two compartments mimicking human intestine (Ekmekcioglu, 2002). In the human gut, nutrients are transported by a transcellular and/or paracellular route (Figure 2.1) from the intestinal lumen to the interstitial fluid and afterwards to the blood. The paracellular transport is accomplished by passive diffusion, which mainly depends on the concentration gradient between the intestinal lumen and the interstitium (Ekmekcioglu, 2002). Other factors like transepithelial potential difference, the pore radius of the tight junctions at the apical poles of the cells and the molecular size of the substances influence passive paracellular transport of nutrients (Ekmekcioglu, 2002).

Over 90 % of the peptides absorbed via transcellular route are further hydrolyzed in the absorptive cells (Heyman & Desjeux, 1992). For example, the intestinal brush border membrane is rich in aminopeptidases, which cleave N- terminal amino acids of peptides. Aminopeptidase N and A are found in intestinal brush border and cleave N-terminal neutral and anionic amino acids, respectively. In addition to these, the intestinal brush border contains endopeptidases and dipeptidases (Vermeirssen *et al.*, 2004). Therefore, the peptides absorbed can be further hydrolyzed by the brush border enzymes degrading them into a mixture of amino acids, di- and tri- peptides. The paracellular pathway of peptide absorption is known as a non degradative transport route, which keeps the transported peptide intact. Whichever the route, peptides longer than di- or tri- peptides even in small amounts could get into the body and demonstrate physiological functions (Shimizu, 2004). In the studies based on membrane dialysis, passive diffusion is considered as the way for nutrients to move between the luminal and serosal chamber (Miller *et al.*, 1981). It is important to note that intestinal brush border enzymes were not used in the present study and the dynamic model represented the absorption of peptides only via passive diffusion.

4.1.5.2.1 The amino acid composition of dialysate and retentate of gastric + intestinal digest of flaxseed protein

Table 4.14 shows the amino acid profile obtained for the dialysate and retentate of the gastric + intestinal digest obtained from the dynamic model of flaxseed protein digestion. The amino acid profile for the gastric digest in the dynamic model was the same as that of the static model and therefore is not shown in Table 4.14.

A comparison was made between the amino acid profile of dialysate (absorbable fraction) and retentate (non-absorbable fraction) as well as between that of gastric + intestinal digests of flaxseed protein obtained from the static and dynamic models. The dialysate and retentate showed considerable differences in terms of their amino acid profile. The dialysate had higher levels of Met, Ser, Ala, Leu, Tyr, Phe, Lys, Arg and Trp compared to that of the retentate (Table 4.14). The dialysis bag, which simulated the intestinal lumen, was expected to contain a mixture of peptides and amino acids generated from flaxseed protein digestion. It was hypothesized that the dialysate had amino acids and peptides with a potential to absorb via passive diffusion, whereas the retentate consisted of peptides and amino acids that could not be absorbed via the small intestinal epithelium. The results showed a clear difference between the amino acid composition of absorbable and non-absorbable fractions during the 4 h intestinal digestion. Interestingly, the amino acid profile of gastric + intestinal digest obtained from the static model was different than that of the dynamic model. A major difference was the presence of the aromatic amino acid Tyr in the digests of the dynamic model, which was absent in the digest of the static model (Table 4.14).

The dynamic model of GI digestion is one step closer to the human digestive system compared to that of the static model. As stated by Savoie (1994), in simulating GI digestion *in vitro*, the removal of digestion products is of prime importance since their accumulation is likely to affect the efficiency and rate of hydrolysis of the substrate. The proteolysis and absorption processes that occur in the small intestine are simultaneous.

Table 4.14 Amino acid composition (g/100 g protein) of gastric + intestinal digest of flaxseed protein obtained from the dynamic model in comparison with the static model

Amino acid	Static model	Dynamic model	
		Dialysate	Retentate
<u>Essential</u>			
His	2.65	1.86	1.93
Ile ¹	4.22	3.93	4.46
Leu ¹	5.57	6.20	4.34
Lys	2.76	3.72	2.71
Met	1.64	1.86	1.29
Phe	5.34	7.02	3.56
Thr	3.49	3.51	3.80
Trp	1.17	2.07	0.98
Val ¹	5.39	5.17	5.29
<u>Non –essential</u>			
Ala	4.43	4.55	4.24
Arg ²	11.38	18.8	10.57
Asp	11.13	8.88	13.79
Cys	2.42	0.62	1.59
Glu	22.72	13.43	20.9
Gly	6.81	4.75	6.27
Pro	2.28	2.69	4.14
Ser	5.28	5.99	5.85
Tyr	0.00	4.13	2.13

¹Branched chain amino acids; ²Conditionally essential (Food and Nutrition Board, 2005)

As hydrolysis proceeds, enzymes, mainly endopeptidases, are constantly exposed to different substrates that vary from complex proteins to oligopeptides of smaller sizes. Kinetics of these enzymatic reactions are favored when the reaction products are removed from the medium. In the static model, the digested products were not removed from the reaction medium. The accumulated digested products could inhibit the action of enzymes thereby decreasing the degree of proteolysis in the intestinal phase (Gauthier *et al.*, 1982). The dialysis allows simultaneous elimination of digested products and reduces the possibility of enzyme inhibition by products (Gauthier *et al.*, 1986). Therefore, continuous elimination of digested products by dialysis in the dynamic model was expected to eliminate the influence of products on the enzyme activity. As a consequence, a higher degree of proteolysis occurred than with the static model (Table 4.9 of section 4.1.3.2.2). The improved efficiency of digestive enzymes in the dynamic model, which led to increased proteolysis could be the major reason for the differences in amino acid composition compared to the static model.

4.1.5.2.2 Degree of hydrolysis and average peptide chain length of digested flaxseed protein

The DH of intestinal digest obtained from the dynamic model was calculated by taking the free amino groups in both dialysate and retentate into consideration. According to Savoie (1994), a higher DH was expected in the dynamic model of intestinal digestion than that of the static model. In the dynamic model, the DH was $46.78 \pm 0.3 \%$, whereas the average PCL was 2.1. The DH obtained for the gastric + intestinal digest of flaxseed protein in the dynamic model was greater than that of the static model ($43.95 \pm 0.8\%$, Table 4.9) as expected. However, the average PCL calculated based on the DH was still around 2.

4.1.5.2.3 Bioactivities of the digested flaxseed protein

When flaxseed protein was digested using the dynamic model, both dialysate and retentate were analyzed for the bioactivities. The results shown for the bioactivities of dialysate and retentate are the net values obtained after deducting the values of enzyme and sample blanks from the sample values. The dialysate was in a large volume of

buffer. Therefore, the freeze dried dialysate had a significantly lower N % (0.77 ± 0.0 %) than that of the retentate (7.96 ± 0.0 %). Due to this reason the IC_{50} values for dialysate and retentate were calculated based on the N % to provide a more accurate comparison.

(a) Angiotensin I-converting enzyme inhibitory activity

Both dialysate and retentate obtained from flaxseed protein digestion had ACEI activity (Table 4.15). Interestingly, the presence of ACEI activity in the dialysate showed that the ACEI peptides in gastric + intestinal digest of flaxseed protein have potential to be absorbed through the small intestine making them bioavailable. The ACEI peptides in the retentate had higher molecular mass (>1 kDa) and did not pass through the dialysis membrane. ACEI activity in the retentate indicated that GI digestion of flaxseed protein releases ACEI peptides with different molecular masses. Of those, the ACEI peptides having <1 kDa may have the potential to be absorbed through the small intestinal wall.

The freeze dried dialysate had lower N levels (0.77 ± 0.0 %) compared to the retentate (7.96 ± 0.0 %) and gastric digest (10.68 ± 0.1 %). Therefore, a higher concentration of dialysate (6.85 mg/mL) was used in the assay mixture for screening of ACEI activity compared to that of the retentate and gastric digest (0.67 mg/mL) to obtain equal N levels. The calculated IC_{50} values were based on N content (mg N/mL) and not the weight concentration of the digests. The IC_{50} values also showed that the overall ACEI potential was numerically greater in the dialysate when compared to the retentate (Table 4.15). However, the results were not significantly different ($P<0.05$). The retentate showed higher, but not significantly different ACEI activity than the dialysate. Although the ACEI activity of both dialysate and retentate were significantly lower ($P<0.05$) than the gastric digest at the used concentration, the IC_{50} values indicated that dialysate and retentate (IC_{50} : 0.04 ± 0.0 and 0.05 ± 0.0 mg N/mL, respectively) had significantly higher ($P<0.05$) ACEI potency than that of gastric digest (IC_{50} : 0.16 ± 0.0 mg N/mL) (Table 4.15). This was in contrast to the static model, which showed lower ACEI potency of gastric + intestinal digest (IC_{50} : 0.39 ± 0.0 mg N/mL) than the gastric digest (IC_{50} : 0.16 ± 0.0 mg N/mL) (Table 4.10).

Table 4.15 Angiotensin I-converting enzyme inhibitory activity of digested flaxseed protein (dynamic model) in relation to average peptide chain length

Sample	Average PCL	ACEI activity (%) ¹	IC ₅₀ (mg of N/mL) ¹
Initiation of digestion	30.2 ^a	0	N.D.
Gastric digest	12.6 ^b	81.32 ± 1.9 ^a	0.16 ± 0.0 ^a
Gastric + Intestinal digest, retained fraction, >1 kDa	2.1 ^c	59.12 ± 2.0 ^b	0.05 ± 0.0 ^b
Gastric + Intestinal digest, dialyzed fraction, <1 kDa ²		52.96 ± 1.8 ^b	0.04 ± 0.0 ^b

¹Values are means ± standard deviations of duplicate analyses

^{abc}Means in the same column followed by the same letters are not significantly different ($P < 0.05$)

²Concentrations of the digests was 0.67 mg/mL in the final assay mixture except for gastric + intestinal digest, dialyzed fraction (6.85 mg/mL)

PCL: Peptide chain length; ACEI: Angiotensin I-converting enzyme inhibitory, N.D.: Not determined

The static model of digestion leads to accumulation of hydrolysis products that may impede the progression of hydrolysis due to enzyme inhibition by products (Stahmann & Woldegiorgis, 1975; Gauthier *et al.*, 1986). Dialysis used in the dynamic model allowed simultaneous elimination of digested products and reduced the possibility of any enzyme inhibition by the generated products. Therefore, differences were expected in the kinetics of digestive enzymes used in the intestinal phase of the static model and the dynamic model. The reduced activity of proteases in pancreatin caused by accumulated digestion products could have caused the hydrolysis of gastric phase generated ACEI peptides within the ACEI sequence, reducing their ACE activity. In the dynamic model, continuous removal of digestion products could have enhanced the activity of pancreatic proteases, especially chymotrypsin, which cleaves peptide bonds at the carboxylic end of aromatic and hydrophobic amino acids (Nelson & Cox, 2000). This may have generated peptides with C-terminal aromatic and hydrophobic amino acids that are reported as competitive ACE inhibitors leading to improved ACEI activity in the gastric + intestinal digest of the dynamic model. The results of the current study further showed that the commonly used two stage proteolysis with no absorption for simulated GI digestion underestimates the ACEI activity of digested food protein.

The IC₅₀ values showed that the ACEI potency of the dialysate (0.04 mg N/mL; 0.23 mg of protein/mL; 4.77 mg of solids /mL) was within the range reported for several cheese varieties (0.16-27.6 mg of solids/mL; Sieber *et al.*, 2010) and similar to that of *in vitro* GI digested proteins from amaranth seeds (IC₅₀: 0.44 mg protein/mL; Tiengo *et al.*, 2009). However, the ACEI potency of the dialysate was lower than that of isolated protein from soy (IC₅₀: 0.28-0.38 mg of solids/mL; Lo *et al.* 2006), pea (IC₅₀: 0.076 mg of solids/mL; Vermeirssen *et al.*, 2003) and whey (IC₅₀: 0.048 mg of solids/mL; Vermeirssen *et al.*, 2003) digested by simulated GI digestion using static digestion models. The present study indicated that the ACEI activity of *in vitro* GI digests could differ with the type of digestion model used therefore comparison of IC₅₀ value of dialysate of the dynamic model with protein digests obtained from static digestion models may not be appropriate.

The study of Akillioglu & Karakaya, (2009) reported poor ACEI activity of the dialysates of common bean (IC₅₀: 0.83 mg protein/mL), pinto beans (IC₅₀: 0.69 mg protein/mL) and green lentil (IC₅₀: 0.89 mg protein/mL) protein obtained from a dynamic digestion model consisted of 6-8 kDa MWCO dialysis membrane. Considering the present study and these reported values, the absorbable flaxseed protein digest may be a better source of ACEI peptides than these legume proteins.

The % ACEI activity of both dialysate and retentate was plotted against their concentrations as shown in Figure 4.7. Both dialysate and retentate showed a dose dependent relationship with ACEI activity, whereas the % ACEI activity increased with increased concentration. At low concentrations, the ACEI activity of both dialysate and retentate was similar. However, at higher concentrations, the ACEI activity of retentate increased at a slower rate compared to that of dialysate (Figure 4.7).

To exert physiological effects *in vivo* after oral ingestion, it is of crucial importance that ACEI peptides retain their activity during GI digestion and absorption and reach the cardiovascular system (Vermeirssen *et al.*, 2004).

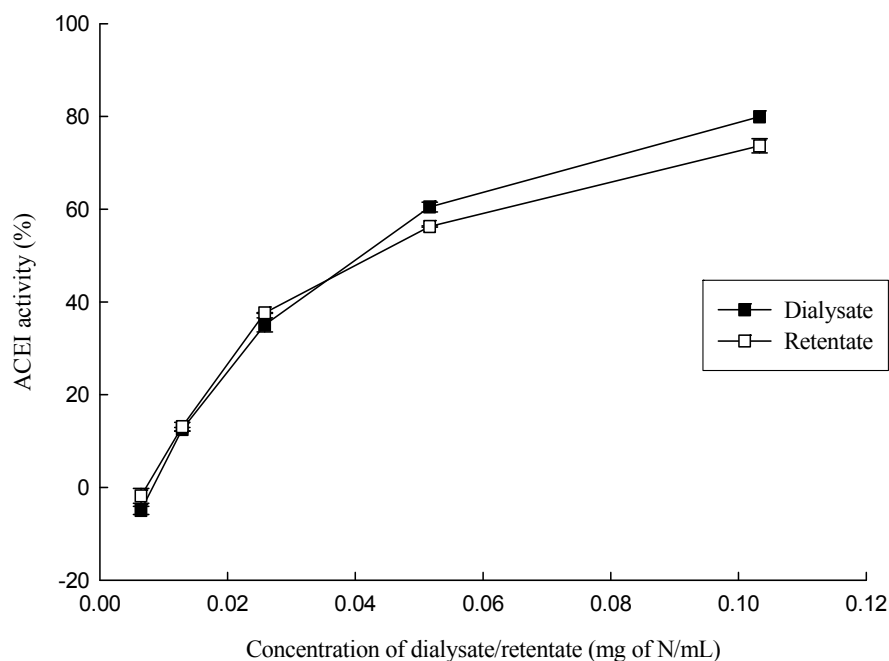


Figure 4.7 Angiotensin I-converting enzyme inhibitory activity of dialysate and retentate of gastric + intestinal digest of flaxseed protein obtained using the dynamic model in relation to the digest concentration in the assay mixture

Most of the studies on bioavailability of ACEI peptides (*in vitro* and *in vivo*) are carried out using milk protein derived peptides. The *in vitro* studies used Caco-2 cell monolayers as models for the small intestine epithelium to express many intestinal enzymes and transport mechanisms (Lopez Fandino *et al.*, 2006). Of the studies showing *in vivo* bioavailability of ACEI peptides, Masuda *et al.* (1996) detected the ACEI peptide Val-Pro-Pro in the abdominal aorta of SHR after administering sour milk. This suggested the ability of Val-Pro-Pro to be transepithelially transported. Satake *et al.* (2002) reported that a significant amount of Val-Pro-Pro is transported via Caco-2 cells. Since the transport via the short-peptide carrier, PepT1, led to a quick hydrolysis of the internalized peptide, they suggested paracellular transport through the intercellular junctions as the main mechanism for absorption of ACEI peptide Val-Pro-Pro via the intestine. Several milk protein derived peptides have been tested for antihypertensive effect in humans. Reduction in blood pressure after consumption of ACEI peptide containing commercial milk products such as Calpis™ (sour milk)

(Mizushima *et al.*, 2004), Ameal peptide[®] (casein hydrolysate produced with *Aspergillus oryzae* containing Ile-Pro-Pro and Val-Pro-Pro) (Mizuno *et al.*, 2005) and Evolus[™] (milk fermented with *Lactobacillus helveticus*) (Seppo *et al.*, 2003) have been proven using human subjects. Two other commercial products, a casein hydrolysate containing the peptide Phe-Phe-Val-Ala-Pro-Phe-Glu-Val-Phe-Gly-Lys (α_{s1} -casein f23-34) and a whey protein hydrolysate have also claimed lowering of blood pressure in humans (FitzGerald *et al.*, 2004).

The bioavailable potential shown by ACEI flaxseed protein digest as shown in the present *in vitro* study is a good starting point for *in vivo* experiments and clinical trials focussing on antihypertensive effect of flaxseed protein.

(b) Hydroxyl radical scavenging activity

When flaxseed protein was digested using the static model where there was no simulated absorption, only the gastric + intestinal digest showed OH[•] scavenging activity ($45.56 \pm 1.1\%$, Table 4.12). In contrast to the static model, OH[•] scavenging activity of the gastric + intestinal digest of flaxseed protein was absent in the dialysate and the retentate obtained from the dynamic model. This may be due to further alteration or cleavage of the OH[•] scavenging peptide sequence resulting from changes in pancreatin digestion in the dynamic model.

Previous studies on hydrolysates from Allaska pollack skin gelatin (Kim *et al.*, 2001), bovine serum albumin (Hatate *et al.*, 1990) and soy protein (Chen *et al.*, 1996) indicate that antioxidant peptides exhibit synergistic effect with non- peptidic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tocopherol. Chen *et al.* (1996) demonstrated that peptides with no antioxidative activity enhance antioxidative activity of non-peptidic antioxidants due to synergistic effects when in a mixture. Therefore, another hypothesis could be the OH[•] scavenging activity of gastric + intestinal digest obtained from the static model was due to the synergistic effect between peptides and a non-peptide antioxidant, which was fractionated in the dynamic model. However, there was no OH[•] scavenging activity in the undigested flaxseed

protein (Table 4.12). Therefore, it showed that the isolated flaxseed protein used for the digestion studies did not contain any non-peptidic antioxidants unless formed as a result of flaxseed protein digestion.

Hayes *et al.* (1977) reported that amino acids such as Cys and Met can act as antioxidants or prooxidants depending on the environment. Bishov and Henick (1975) reported the synergistic antioxidant activity between peptides and amino acids when present in a mixture. In the present simulated GI study (both static and dynamic models), the gastric + intestinal digests were expected to contain a mixture of amino acids and peptides. In the dynamic model, amino acids were expected to be in the dialysate with peptides <1 kDa. This separation may have caused the loss of any synergistic effect between peptides and amino acids on OH[•] scavenging activity. Therefore, it is suggested that separation of amino acids from peptides in the dynamic model could be the reason for the loss of OH[•] scavenging activity of gastric + intestinal digest. Another possible reason could be the differences in the enzyme kinetics between the static and the dynamic models. Removal of products from the hydrolytic reaction in the dynamic model may have allowed the cleavage of the retained larger peptides further into small peptides with sequences different than that obtained from the static model.

Release of antioxidant peptides by oilseed proteins such as soy (Chen *et al.*, 1995, 1998; Park *et al.*, 2010), peanut (Chen *et al.*, 2007) and rapeseed (Xue *et al.*, 2009) have been reported. Moreover, recent studies have reported antioxidative activity of flaxseed protein hydrolysates (Marambe *et al.*, 2008; Udenigwe *et al.*, 2009; Udenigwe *et al.*, 2010). None of these are simulated GI digestion studies. Besides that, the proteins have been hydrolyzed using commercial proteases of microbial origin or by using a single digestive enzyme in these studies. There are *in vitro* studies reporting the ability of fish proteins to generate antioxidant peptides upon digestion with digestive enzymes found in the GI tract (Kim *et al.*, 2007; Nakajima *et al.*, 2009). These simulated *in vitro* GI digestion studies are based on the static digestion models without simulating absorption process. The results of the present study indicate that the peptides generated using the

static model will be different than when absorption is simulated, thereby influencing the bioactivity of the resulting hydrolysis product. Since the dynamic model closely mimics the human GI digestion, results obtained from the dynamic model were considered closer to the *in vivo* condition than the static model. The ability to scavenge OH[•] was shown only by the gastric + intestinal digest obtained from the static model but not from the dynamic model. Therefore, it could be hypothesized that bioactive peptides with OH[•] scavenging activity are not generated during GI digestion of flaxseed proteins. Considering the inability to scavenge OH[•], the digests obtained using the dynamic model were not further studied for antioxidant activity. However, the findings from the static model exhibited that *in vitro* digestion of isolated flaxseed protein with two step hydrolysis using pepsin followed by pancreatin can generate a hydrolysate with OH[•] scavenging activity. This information will be important in developing functional food ingredients such as antioxidant flaxseed protein hydrolysate with peptides resistant to further hydrolysis by digestive enzymes.

4.1.5.3 Flaxseed protein degradation pattern during simulated gastrointestinal digestion and its relationship with bioactivities of the digests

The degradation pattern of polypeptides in the isolated protein during simulated GI digestion (static and dynamic models) was assessed using SDS-PAGE profile. Figure 4.8 and 4.9 show the electrophoretic profile of the unhydrolyzed protein and the remaining residues of flaxseed protein upon simulated GI digestion using the static and dynamic models.

A clear difference in polypeptide degradation pattern was not observed between the SDS- PAGE profile of the remaining residues (NR) for the static and dynamic models. When separated proteins were analyzed for molecular weights, five major polypeptide bands (~ 48, 41, 29, 20 and 13 kDa) were visible in the isolated flaxseed protein under NR conditions (Figures 4.8a, b and 4.9b). At the end of gastric phase digestion of both models, the polypeptide bands within the molecular mass range of ~ 20 to 48 kDa disappeared completely leaving the low molecular mass bands that also showed a reduction in the intensity (Figure 4.9c and Table 4.16).

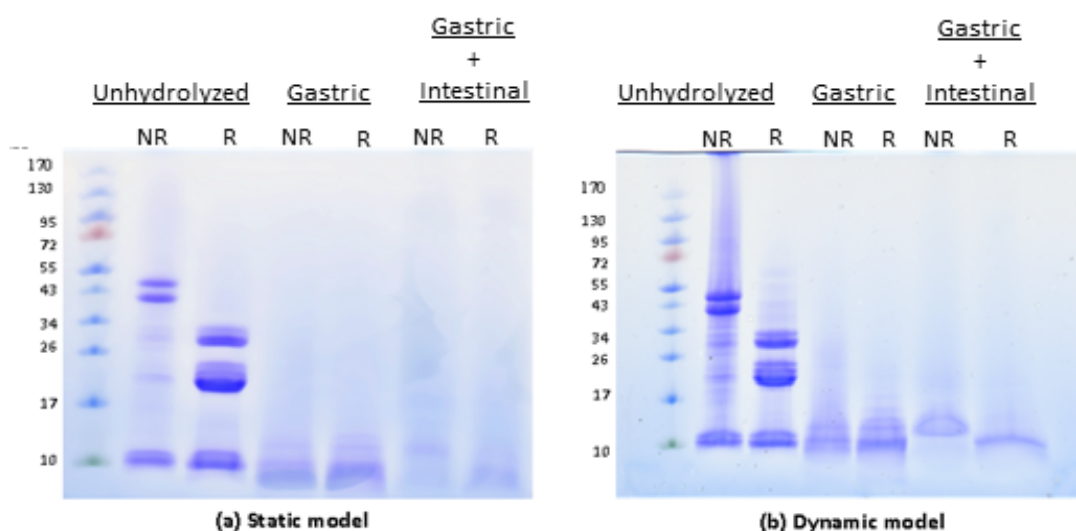


Figure 4.8 SDS PAGE profile of the unhydrolyzed protein and remaining residue of flaxseed protein obtained from (a) static model and (b) dynamic model (R: reducing, NR: non reducing). Gels used were gradient mini gels (resolving 8-25% T and 2% C, stacking zone 4.5 %T and 3 %C, 43×50×0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4). Each well was loaded with 5 µg protein

At the end of subsequent intestinal phase digestion, the LMW band (~13 kDa) showed a further reduction of intensity, which was greater in the static model compared to that of the dynamic model as shown in Figure 4.9d. Due to the significantly higher DH value shown, a higher percentage loss of pixel intensity was expected in the remaining residue from gastric + intestinal digestion of the dynamic model compared to the static model. Moreover, there was appearance of a new band below 13 kDa at the end of gastric + intestinal digestion and this was mainly visible in the static model. These results revealed that there is a difference in protein hydrolysis when intestinal phase was simulated with simultaneous absorption compared with no absorption. This is due to the influence of absorption of digested products on the enzyme kinetics as described by Savoie (1994).

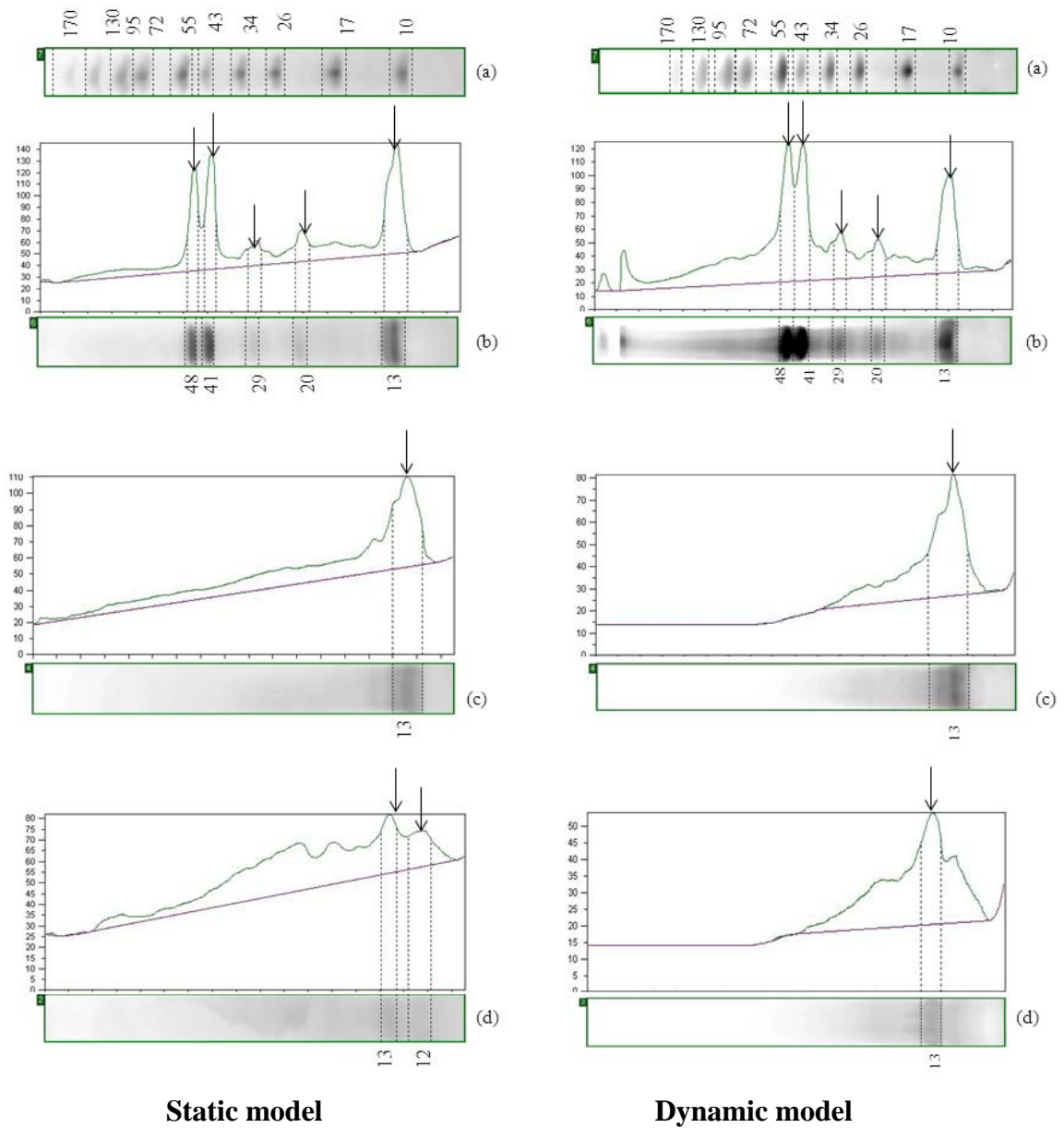


Figure 4.9 Electropherogram images (X axis: Pixel position; Y axis: Pixel intensity) of polypeptide bands in the unhydrolyzed protein and the remaining flaxseed protein residue (non-reducing) at the end of each digestion phase using the static and the dynamic models of digestion. (a) Molecular weight markers (kDa), (b) Initiation of digestion, (c) at the end of gastric phase (2 h), (d) At the end of gastric + intestinal phase (6 h)

Table 4.16 Difference (%) in polypeptide band intensity in the remaining flaxseed protein residues at the end of digestion phases compared to the initiation of digestion

Model	Digestion phase	Percentage difference in pixel intensity ¹ of polypeptide bands				
		48 kDa	41 kDa	29 kDa	20 kDa	13 kDa
Static	Gastric (2 h)	-100	-100	-100	-100	+14.8
	Gastric + intestinal (6 h)	-100	-100	-100	-100	-80.6
Dynamic	Gastric (2 h)	-100	-100	-100	-100	+20.5
	Gastric + intestinal (6 h)	-100	-100	-100	-100	-41.8

¹Negative values indicate reduction of pixel intensity and positive values indicate gaining of pixel intensity of polypeptide bands compared to the initiation of digestion

Accumulation of insoluble degradation products of the LMW polypeptide band during gastric + intestinal digestion could be suggested as the reason for the appearance of a new polypeptide band <13 kDa in the residue obtained at the end of gastric + intestinal digestion. However, the LMW band was not completely hydrolyzed at the end of intestinal phase digestion of both models. The degradation pattern of flaxseed protein (static model) at each phase of digestion is discussed in detail in section 4.2.3 of the results and discussion chapter of this thesis.

The results showed that digestion with pepsin was able to hydrolyze all the major protein bands in the isolated flaxseed protein. The HMW bands were extensively hydrolyzed by pepsin. The LMW polypeptide bands remaining upon pepsin catalyzed digestion were further hydrolyzed by pancreatin. Based on the SDS- PAGE profiles of undigested residues of flaxseed protein, it could be hypothesized that degradation of polypeptides above 13 kDa may be mainly responsible in generating peptides with high ACEI activity. The hydrolysis of polypeptide of ~13 kDa could be responsible for generation of OH[•] scavenging peptides in the intestinal phase of the static model.

4.1.5.4 Peptide fractionation using size exclusion chromatography

Size exclusion chromatography was used to estimate the molecular mass distribution profile and to fractionate the bioactive flaxseed protein digests into peptides based on their molecular masses. It is widely used as the first step to separate complex mixtures of peptides where the peptides are separated based on the molecular size. Figure 4.10 indicates the size exclusion chromatograph of the standard peptides (Insulin chain A, angiotensin II and Hip- His- Leu) used.

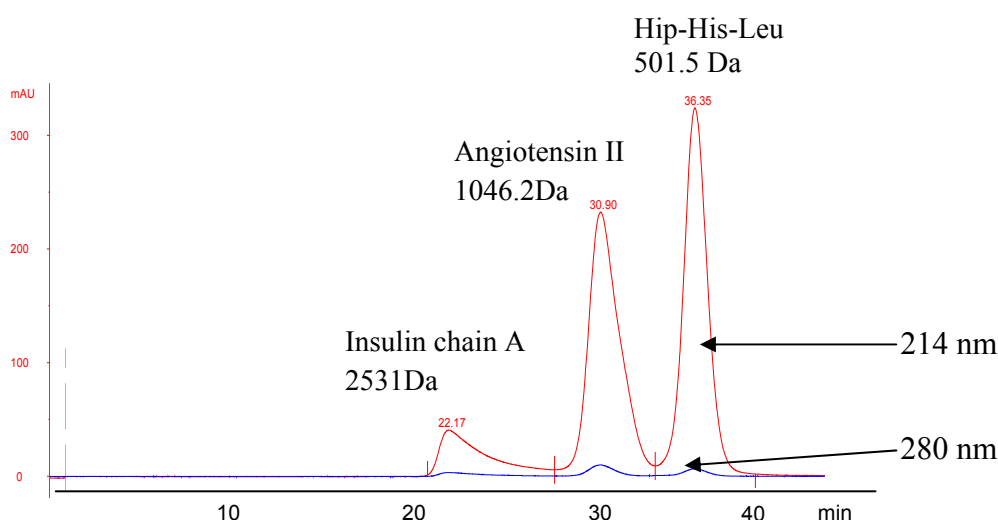


Figure 4.10 Elution profiles of molecular weight standards. Superdex peptide 10/30 was used with the AKTA explorer system. The column was equilibrated and eluted with 20 % (v/v) acetonitrile with 0.05 % (v/v) TFA, at a flow rate of 0.5 mL/min

The absorbance at 214 nm is specific for peptide bonds. Therefore, presence of peptides in the observed peaks (Figure 4.10) was confirmed. The aromatic amino acids, their derivatives, peptides as well as proteins containing aromatic amino acids absorb at 280 nm (Amarowicz & Shahidi, 1997). Therefore, the presence of peptides with Trp, Phe or Tyr was confirmed when higher absorbance was seen at 280 nm. The flaxseed protein digests were injected to the size exclusion column (SEC) and subjected to FPLC under similar conditions to that used for peptides of known molecular weights. The molecular mass distribution profile of peptides in the digests was determined based on the retention times shown by these standard peptides.

4.1.5.4.1 Molecular mass distribution profile of the gastric digest of flaxseed protein

Figure 4.11 shows the molecular mass distribution profile of flaxseed protein digested by pepsin in the simulated gastric phase. The gastric digest was comprised of peptides larger than 0.5 kDa. The presence of high molecular mass peptides (>1.05 kDa) as the majority in the gastric digest further confirmed the observed low DH ($7.96 \pm 0.2\%$) and long average PCL (12.5) values (Table 4.17). It further indicated that pepsin digestion resulted in generation of long chain peptides from flaxseed protein.

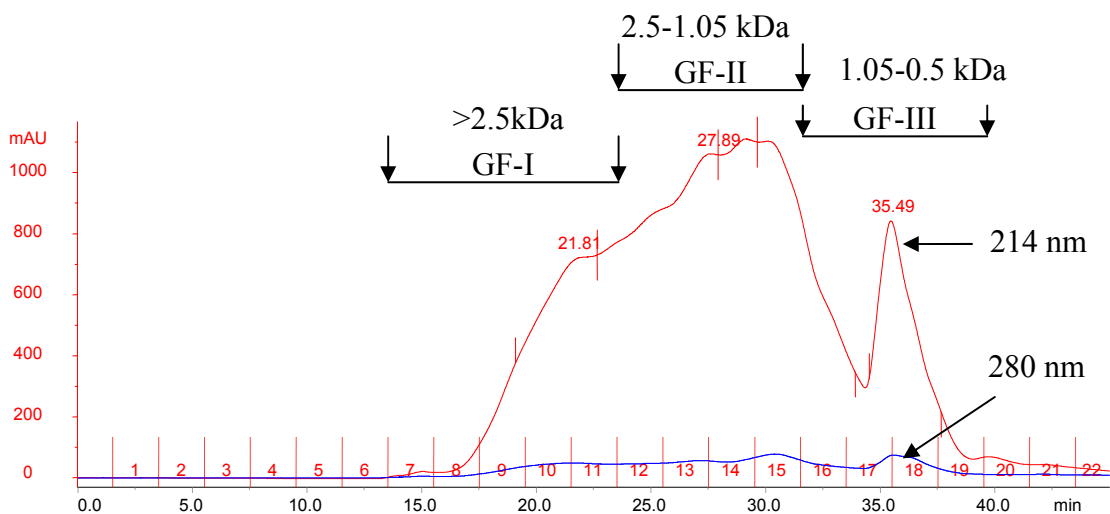


Figure 4.11 Molecular mass distribution profile of peptides in the gastric digests of flaxseed protein

Peptide fractions of the gastric digest were collected based on the elution time of standard peptides, insulin chain A (2.5 kDa), angiotensin II (1.04 kDa) and Hip-His-Leu (0.5 kDa). The obtained fractions were pooled into three fractions; namely, GF-I (>2.5 kDa), GF-II (1.05-2.5 kDa) and GF-III (0.5-1.05 kDa) (Figure 4.11). GF-II and GF-III yielded very low absorbance at 280 nm. Therefore, the peptides in these fractions may contain aromatic amino acids at very low levels. Absence of any peak at 280 nm indicated the lack of aromatic amino acids in GF-I.

(a) Angiotensin I-converting enzyme inhibitory activity of peptide fractions in the gastric digest

As the unfractionated gastric digest did not show any OH[•] scavenging activity, the peptide fractions were not analyzed for OH[•] scavenging activity. Only the ACEI activity of these fractions was determined. The peptides in GF-I (>2.5 kDa) had no ACEI activity whereas both GF-II (1.05-2.5 kDa) and GF-III (0.5-1.05 kDa) exhibited ACEI activity (Table 4.17). Of the peptides possessing ACEI activity, those between 0.5 to 1.05 kDa had significantly higher ($P<0.05$) ACEI activity than those between 1.05 to 2.5 kDa (Table 4.17). This indicates that the ACEI activity of peptides in the gastric digest increases with the decrease in the size of the peptides. The results further suggest that the ACEI activity of the unfractionated gastric digest was due to peptides with molecular masses between 1.05 to 0.5 kDa. The GF-III fraction had the highest ACEI activity and the IC₅₀ value was 0.07 ± 0.0 mg/mL (Table 4.17). This value was lower than that of unfractionated gastric digest, which was 0.25 ± 0.0 mg/mL (Table 4.10) indicating that separation of small and large peptides from the gastric digest resulted in segregation of ACEI molecules from the complex mixture.

Table 4.17 Angiotensin I-converting enzyme inhibitory activity of gastric digested flaxseed protein fractions in relation to their molecular masses

Fraction	Molecular mass of peptide fraction	ACEI (%) ¹	IC ₅₀ (mg of solids /mL) ¹
GF-I	>2.5kDa	-	-
GF-II	1.05-2.5 kDa	82.92 ± 2.8^b	N.D.
GF-III	0.5-1.05 kDa	99.44 ± 0.1^a	0.07 ± 0.0

¹Values are means \pm standard deviations of duplicate analyses

^{ab}Means in the same column followed by the same letter are not significantly different ($P<0.05$)

ACEI: Angiotensin 1-converting enzyme inhibitory, GF: Gastric digest fraction

N.D.: Not determined

In a recent study by Udenigwe *et al.* (2009), an IC₅₀ value between 0.04 and 0.06 mg/mL for LMW fraction (<1 kDa) of pepsin digested flaxseed protein was identified.

These values are lower than that found in the present study (0.07 ± 0.0 mg/mL, Table 4.17). Udenigwe *et al* (2009) utilized an E/S of 1/100 and digestion time of 4 h with pepsin, which provided greater enzyme level and longer digestion time compared to that employed in the present study (E/S: 1/250 w/w, digestion time 2 h). Therefore, the size /length of peptides generated by the action of pepsin on flaxseed protein would be different in these studies causing the differences in ACEI activities. Moreover, the specific enzyme activity of pepsin was not specified by Udenigwe *et al.* (2009), which made the comparison between the findings of their study with that of ours more difficult.

4.1.5.4.2 Molecular mass distribution profile of the gastric + intestinal digest of flaxseed protein and bioactivities of the peptide fractions-Static model

The gastric + intestinal digest of flaxseed protein contained a negligible amount of peptides >2.5 kDa (Figure 4.12, peptides eluting at a retention time of 22 min or less). The majority of the peptides in the digest had molecular masses ranging from 2.5 kDa to <0.5 kDa. This was also in accordance with Lo and Chan (2005), who reported that digestion of soy protein isolate with pepsin followed by pancreatin released peptides <3 kDa molecular mass. Simulated GI digestion of chickpea (Kabuli and Desi) and yellow pea protein resulted in peptides with molecular weight <4 kDa (Barbana & Boye, 2010). The peptides eluting with a retention time less than 40 min was found to be < 0.5 kDa based on the molecular weight distribution profile of standards (Figure 4.10).

The gastric + intestinal digest obtained using the static model was fractionated into two major fractions of >1 kDa (sGIF-I) and <1kDa (sGIF-II) molecular mass as shown in Figure 4.12 for assessment of bioactivities. Of these, sGIF-II was considered more important than sGIF-I as the smaller peptides (<1 kDa) have a greater potential to be absorbed through the small intestine than peptides with more than three amino acid residues. The majority of peptides in the digest was of <1 kDa molecular mass (Figure 4.12). This was in contrast to the gastric digest in which the majority of peptides were >1 kDa molecular mass.

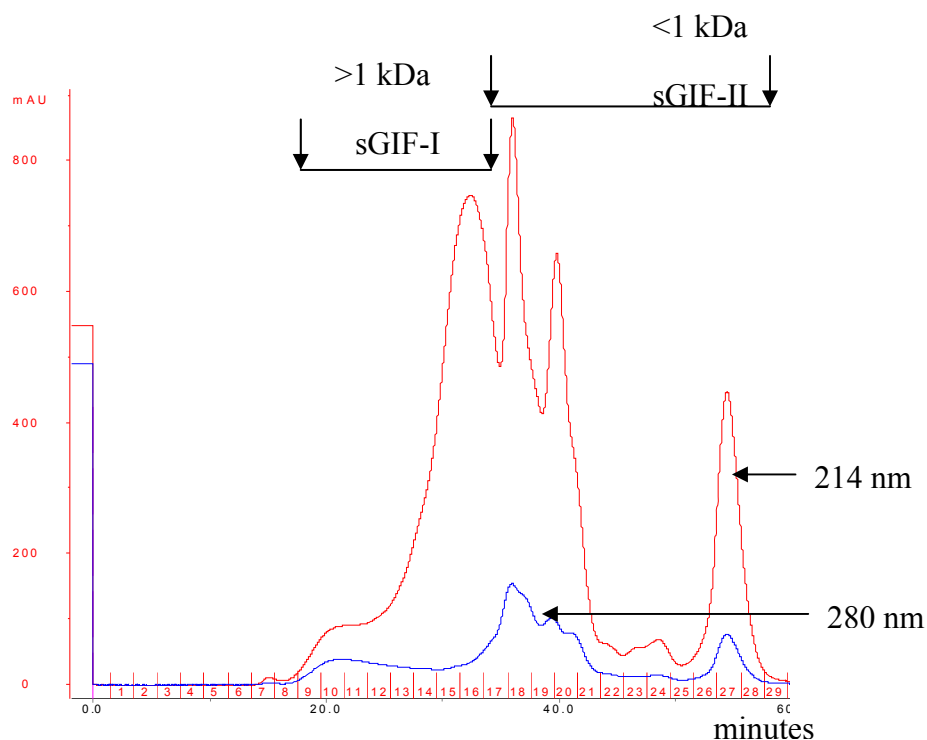


Figure 4.12 Molecular mass distribution profile of peptides in the gastric + intestinal digest of flaxseed protein (static model)

The peptides of low molecular mass eluting around 60 min were present in the gastric + intestinal digest but were not observed in the gastric digest. The results revealed that pancreatic enzymes in the presence of bile salts had broken down the pepsin digested flaxseed protein further into much smaller peptides. The peaks observed at 280 nm in sGIF-II could be due to aromatic amino acid containing peptides (Figure 4.12).

The peptide fractions obtained from simulated gastric + intestinal digestion of flaxseed proteins were considered more important than the gastric digest alone as the small intestinal digestion hydrolyzed the gastric digest further and generated shorter peptides that may be bioavailable. The peptide fractions were further evaluated for both bioactivities because the gastric + intestinal digest before fractionation exhibited ACEI and OH^\bullet scavenging activities.

(a) Angiotensin I-converting enzyme inhibitory activity

According to the results of Table 4.18, the ACEI activity of peptide fractions (sGIF-I and sGIF-II) of the gastric + intestinal digest (static model) was found to be greater than that of unfractionated digest (Table 4.10). The IC_{50} of both fractions were smaller than that of the unfractionated digest indicating the greater ACEI potency of the peptide fractions than the unfractionated digest. Lo and Chan (2005) also reported that fractionation of soy protein digests resulted in a higher ACEI active peptide fraction than crude unfractionated digest.

Table 4.18 Angiotensin I-converting enzyme inhibitory activity of peptide fractions in the gastric + intestinal digest obtained using the static model

Fraction	Molecular mass of peptide fraction	ACEI (%) ¹	IC_{50} (mg of solids/mL) ¹
sGIF-I	>1 kDa	61.0 ± 0.2^a	0.50 ± 0.0^a
sGIF-II	<1 kDa	54.1 ± 1.7^b	0.55 ± 0.0^a

¹Values are means \pm standard deviations of duplicate analyses

^{ab}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

ACEI: Angiotensin 1-converting enzyme inhibitory, sGIF: Static model gastric + intestinal digest fraction

The sGIF-I (<1 kDa) had significantly higher ($P < 0.05$) ACEI activity than that of sGIF-II (>1 kDa) at the concentration 0.67 mg/mL. However, the IC_{50} of sGIF-I and sGIF-II was not significantly different ($P < 0.05$) (Table 4.18). This showed that both fractions had similar potency for ACE inhibition. Of these, sGIF-II was the most important fraction as it consisted of short chain peptides, which could have greater potential for absorption than large peptides.

The <1 kDa fraction obtained from the hydrolysis of flaxseed protein with pancreatin (E/S: 1/100, time 4 h) and then by ultrafiltration exhibited an IC_{50} of 0.14 to 0.16 mg/mL for ACE inhibition (Udenigwe *et al.*, 2009). This value was smaller than the IC_{50} for <1 kDa fraction observed in the present study (0.55 ± 0.01 mg/mL). Pepsin hydrolyzed flaxseed protein digest was subjected to subsequent digestion with pancreatin to simulate intestinal digestion after the gastric phase, instead of subjecting

undigested protein for pancreatin digestion as Udenigwe *et al.* (2009) reported. A comparison between the results of present study (Table 4.18) and Udenigwe *et al.* (2009) lead to the hypothesis that ACEI potential of flaxseed protein digest could be improved if gastric phase digestion was blocked and flaxseed protein is directed to digestion by the intestinal phase enzymes only. However, we observed a reduction in ACEI activity for the unfractionated digests when flaxseed protein was treated with intestinal phase enzymes without using a gastric phase break down (Table 4.11). The variability in the E/S, which influences the kinetics of enzyme catalyzed hydrolysis, and fractionation of peptides could be the factors that caused the differences in ACEI activities of peptides generated during flaxseed protein digestion by pepsin and pancreatin in these two studies. Also in contrast to the study of Udenigwe *et al.* (2009), bile acids along with pancreatin were used in the intestinal phase of the current study. The bile acids assist in lipid absorption and affect the digestion and assimilation of dietary proteins by accelerating the hydrolysis by pancreatic proteases. Bile acids could bind to hydrophobic pocket(s) of the protein substrates and may destabilize its structure, making additional interior domains of the dietary protein available for luminal endoprotease action (Gasset *et al.*, 2007). This further explains the differences found in the peptides of flaxseed protein digest obtained in our study compared to that of Udenigwe *et al.* (2009).

Figure 4.13 shows the ACEI activity of the unfractionated gastric + intestinal digest (static model) and its peptide fractions in relation to the concentration in the assay mixture. At low concentrations, sGIF-I and sGIF-II had similar ACEI activity. When the concentration exceeded 0.3 mg/mL the ACEI activity of sGIF-I (> 1 kDa) exceeded that of sGIF-II (<1 kDa) and was closer to that of sGIF-II at 1.3 mg/mL. The unfractionated digest showed an ACEI activity lower than that of peptide fractions except at 0.7 mg/mL where it had an ACEI activity similar to sGIF-II (Figure 4.13).

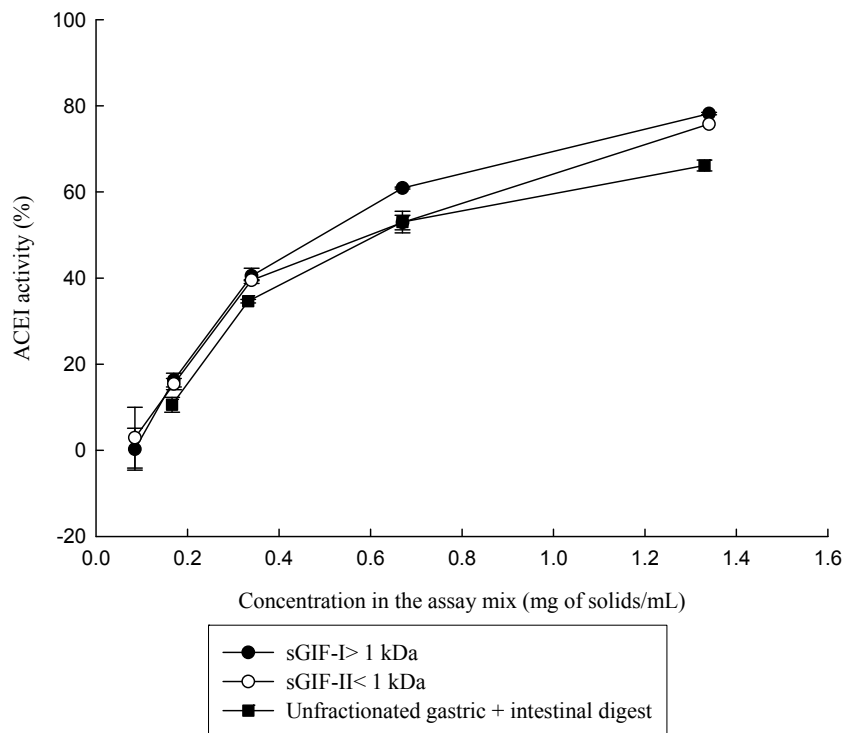


Figure 4.13 Angiotensin I-converting enzyme inhibitory activity of unfractionated gastric + intestinal digest of flaxseed protein (static model) and its peptide fractions in relation to concentration in the assay mixture

(b) Hydroxyl radical scavenging activity

Table 4.19 shows the OH^\bullet scavenging activity of sGIF-I and sGIF-II. The sGIF-II (<1 kDa), which had LMW peptides exhibited significantly higher ($P<0.05$) OH^\bullet scavenging activity ($70.62 \pm 0.4 \%$) than sGIF-I (>1kDa; $55.32 \pm 0.0 \%$) at the concentration used (Table 4.19). However, there was no significant difference between the IC_{50} values of sGIF-I ($0.46 \pm 0.1 \text{ mg/mL}$) and sGIF-II ($0.44 \pm 0.0 \text{ mg/mL}$). Similar to ACEI activity, both fractions showed improved OH^\bullet scavenging activity than that of the unfractionated digest (IC_{50} : $0.70 \pm 0.1 \text{ mg/mL}$; Table 4.12).

Figure 4.14 shows the OH^\bullet scavenging activity of the unfractionated and fractionated gastric + intestinal digests in relation to the concentrations used in the assay mixture. According to Figure 4.14, the OH^\bullet scavenging activity of the fractionated gastric +

intestinal digests apparently deviated from that of the unfractionated digest at high concentrations. The differences were minimal at low concentrations.

Table 4.19 Hydroxyl radical scavenging activity of peptide fractions in the gastric + intestinal digest-Static model

Fraction	Molecular mass of peptide fraction	OH [•] scavenging activity(%) ¹	IC ₅₀ (mg /mL) ¹
sGIF-I	>1 kDa	55.32 ± 0.0 ^b	0.46 ± 0.1 ^a
sGIF-II	<1 kDa	70.62 ± 0.4 ^a	0.44 ± 0.0 ^a

¹Values are means ± standard deviations of duplicate analyses

^{ab}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

OH[•]: Hydroxyl radical, sGIF: Static model gastric + intestinal digest fraction

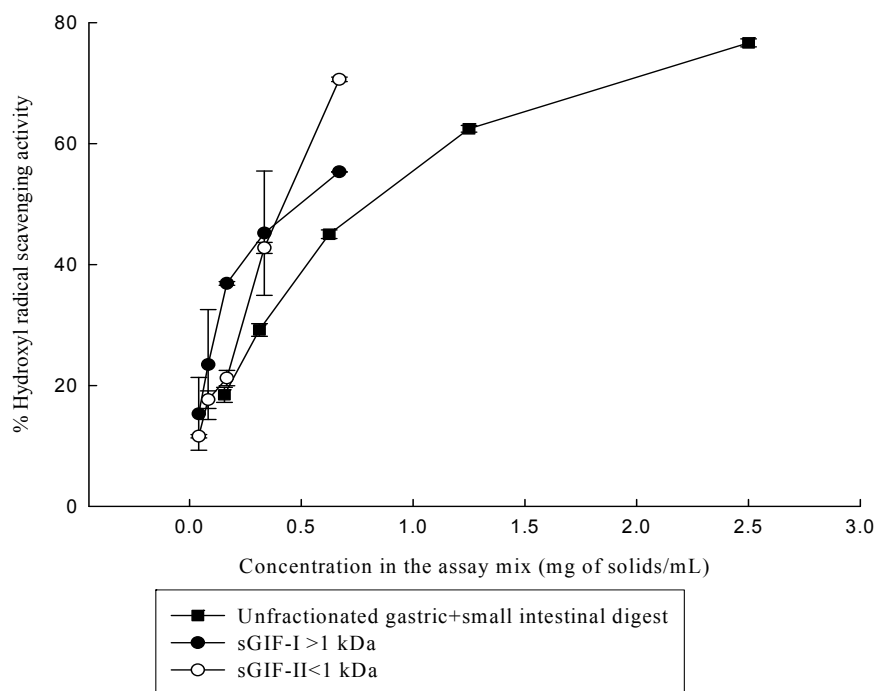


Figure 4.14 Hydroxyl radical scavenging activity of the unfractionated gastric + intestinal digest of flaxseed protein and its peptide fractions in relation to the concentration in assay mixture

Recent reports also have shown that flaxseed protein hydrolysates <1 kDa have OH[•] scavenging activity (Udenigwe *et al.*, 2009). However, in those studies flaxseed protein was subjected to hydrolysis by pepsin and pancreatin separately, which is different from the sequential digestion by pepsin and pancreatin employed in the current study. The lack of OH[•] scavenging activity of unfractionated dialysate obtained using the dynamic model of the current study indicated that the bioavailability of OH[•] scavenging peptides in sGIF-II is unlikely. Therefore, the OH[•] scavenging activity of the fractions of gastric + intestinal digest obtained using the static model was not studied further.

4.1.5.4.3 Molecular mass distribution profile of the gastric + intestinal digest of flaxseed protein and bioactivities of the peptide fractions of dialysate-Dynamic model

Figure 4.15 shows the FPLC chromatograms of retentate (a) and dialysate (b) of gastric + intestinal digest of flaxseed protein obtained using the dynamic model. There was a noticeable difference in the molecular mass distribution profile of both retentate and dialysate as expected. The retentate consisted of both large (>1 kDa) and small (<1kDa) peptides. This shows that although the MWCO of the dialysis membrane was 1 kDa there were notable amount of peptides from flaxseed protein digestion < 1 kDa retained in the dialysis bag without diffusing out during the 4 h of intestinal phase digestion.

The dialysate was expected to have peptides <1 kDa. However, in addition to the <1 kDa peptides, it also had smaller amount of peptides of >1 kDa (Figure 4.15). Although there is no clear explanation for this observation, the > 1 kDa fraction in the dialysate may be a result of aggregation of small peptides during the concentration (freeze drying) step.

Both dialysate and retentate had peptides containing aromatic amino acids (Figure 4.15, Absorbance at 280 nm). In the retentate, aromatic amino acids were found in both peptides greater than and less than 1 kDa of size. However, in the dialysate, aromatic amino acids were mainly confined to peptides of <1 kDa.

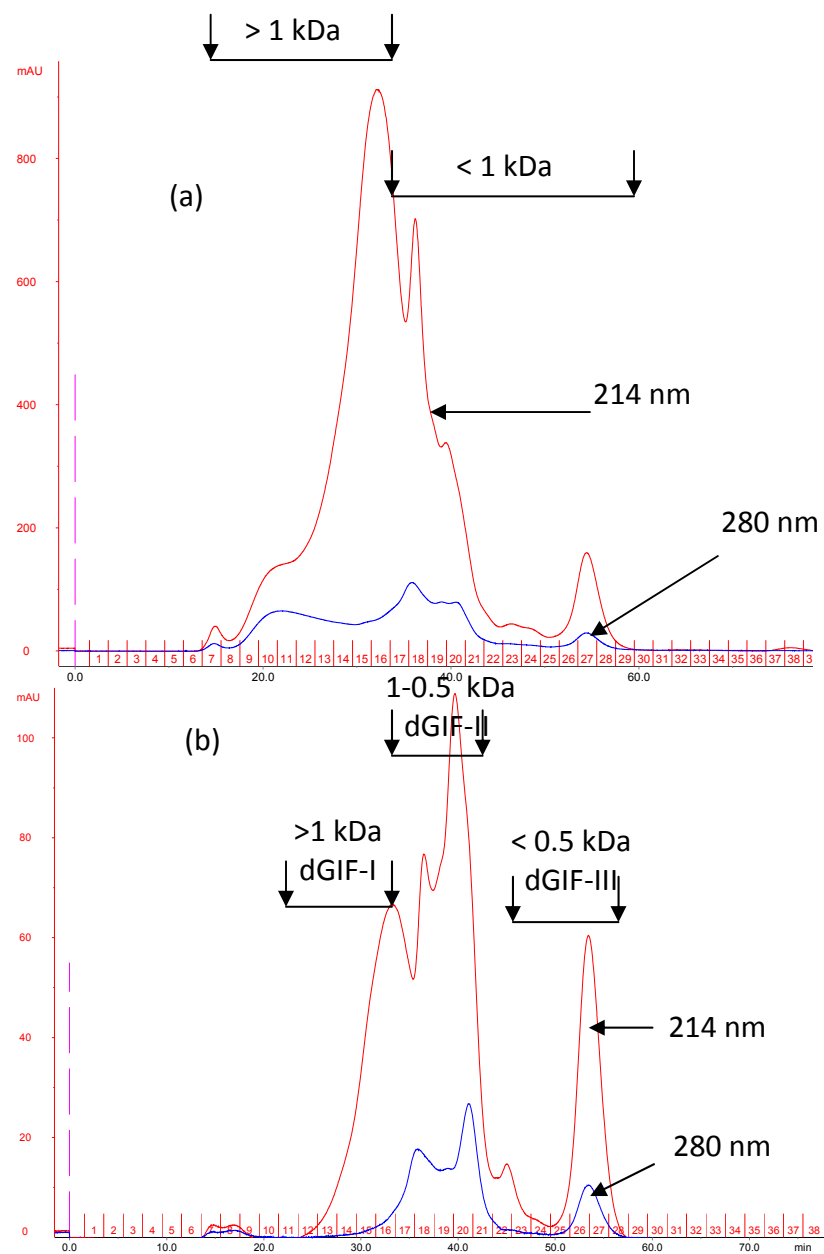


Figure 4.15 Molecular mass distribution profile of peptides in the (a) retentate and (b) dialysate of the gastric + intestinal digest of flaxseed protein obtained using dynamic model

Since the dialysate represents the bioavailable digestion products of flaxseed protein, only the dialysate was fractionated into peptides of different molecular masses (Figure 4.15 b) for further detection of bioactivities. Three fractions were collected from the dialysate. dGIF-I had peptides >1 kDa whereas dGIF-II and dGIF-III had peptides <1kDa (Figure 4.15).

(a) Angiotensin I-converting enzyme inhibitory activity

The percent ACEI activity (at 6.66 mg of solids/mL) and IC₅₀ values for ACE inhibition determined for the peptide fractions of dialysate of gastric + intestinal digest (dynamic model) are shown in Table 4.20. The high molecular mass peptide fraction in the dialysate (dGIF-I) showed the highest ACEI activity (94.96 ± 0.9 %), whereas the fraction with the lowest molecular mass (dGIF-III) had the lowest ACEI activity (1.36 ± 1.4 %) (Table 4.20). However, the fraction dGIF-II which had <1 kDa peptides had considerably high ACEI activity (73.19 ± 0.8 %) about 20 % lower than dGIF-I and 70 % higher than dGIF-III. The IC₅₀ for ACEI activity was determined only for dGIF-I and dGIF-II and was not determined for dGIF-III due to negligible ACEI activity shown by that fraction. The IC₅₀ value of dGIF-II (3.71 ± 0.0mg/mL) was significantly higher (*P*<0.05) than that of dGIF-I indicating significantly higher (*P*<0.05) ACEI activity than that of dGIF-II (Table 4.20).

Table 4.20 Angiotensin I-converting enzyme inhibitory activity of peptide fractions in the dialysate of gastric + intestinal digest-dynamic model

Fraction	Molecular mass of peptide fraction	ACEI activity (%) ¹	IC ₅₀ (mg of solids/mL) ¹
dGIF-I	>1 kDa	94.96 ± 0.9 ^a	0.61 ± 0.0 ^b
dGIF-II	0.5-1 kDa	73.19 ± 0.8 ^b	3.71 ± 0.0 ^a
dGIF-III	<0.5 kDa	1.36 ± 1.4 ^c	N.D.

¹Values are means ± standard deviations

^{abc}Means in the same column followed by the same letter are not significantly different (*P*<0.05)

ACEI: Angiotensin I-converting enzyme inhibitory; dGIF: Dynamic model gastric + intestinal digest fraction

N.D.: Not determined

Figure 4.16 presents the ACEI activity of the unfractionated dialysate and the dialysate fractions plotted against the concentration in assay mixture (mg of solids/mL).

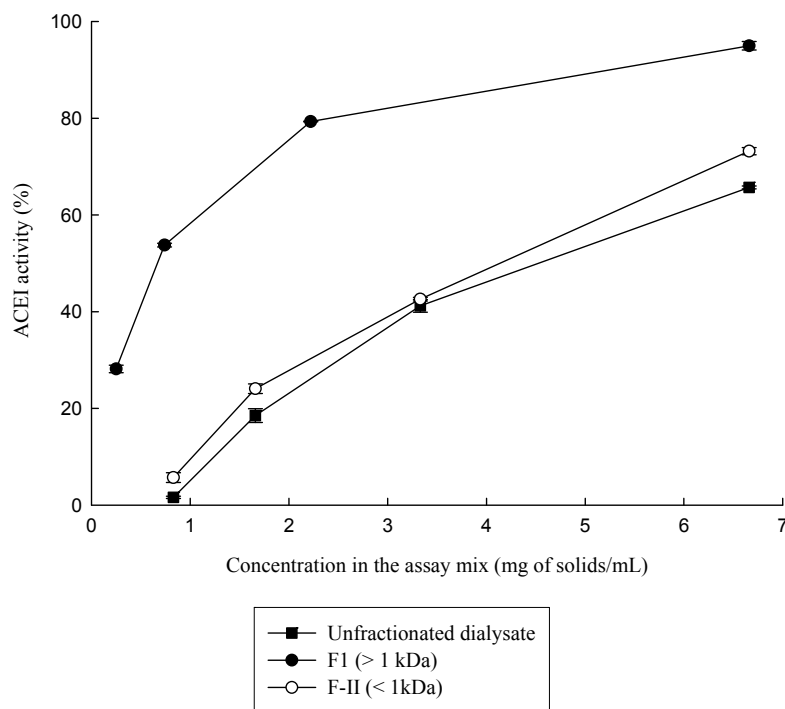


Figure 4.16 Angiotensin I-converting enzyme inhibitory activity of unfractionated dialysate obtained using the dynamic model and its peptide fractions in relation to concentration in assay mixture

The percent ACEI activity of dGIF-I was greater than that of unfractionated dialysate and dGIF-II throughout the range of concentrations studied. However, dGIF-II was selected for further purification and for peptide sequencing as it included molecules <1 kDa, which had absorption potential via the small intestine.

4.1.5.5 Sequence of peptides in the most Angiotensin I-converting enzyme inhibitory fraction of digests

The amino acid sequence of peptides in the most ACEI-FPLC fractions of flaxseed protein digests (GF-III and dGIF-II) obtained using the dynamic model were analyzed. The bioactive FPLC fractions of the gastric + intestinal digest obtained from the static model was not used for sequencing because of the differences in bioactivities observed

between the static and the dynamic model. The peptide content in the FPLC fractions with ACEI activity were first analyzed by ESI-MS connected to a UPLC. The most abundant ions in total ion current chromatograms of each FPLC fraction (results not shown) were selected and subjected to *de novo* sequencing using UPLC-MS/MS. The amino acid sequence and MS/MS spectra of those peptides identified with high certainty (> 50 %) are given in Table 4.21 and Appendix II, respectively.

The amino acid sequence of four peptides in GF-III and six peptides in dGIF-II were identified with high certainty (>50 % probability). The PCL of the sequenced peptides in GF-III and dGIF-II ranged from 6-8 and 5-7, respectively. The molecular mass of peptides in GF-III ranged from 715 to 855.377 Da. Peptides in dGIF-II were in the range of 602 to 874 Da. At present there is no published study reporting the sequence of ACEI peptides released from GI digested flaxseed protein. A search carried out in databases such as Biopep (<http://www.uwm.edu.pl/biochemias>), EROP-Moscow (<http://eropinbi.ras.ru>), SwePep (<http://www.swepep.org>) and PepBank (<http://pepbank.mgh.harvard.edu>), which consist of potentially bioactive peptide sequences, showed that there is no previously reported ACEI peptides possessing the similar sequences of flaxseed protein derived ACEI peptides identified in the present study. However, some of the previously reported ACEI sequences were located within the peptides sequenced in the present study. For example, based on an *in silico* approach, Iwaniak and Dziuba (2009) reported the antihypertensive peptide sequences, Val-Leu-Pro and Val-Phe in soy 7S globulin. The sequence Val-Leu-Pro was also found in chicken connectin and β casein (Iwaniak & Dziuba, 2009). This sequence was found within the peptide Phe-Val-Leu-Pro-Gln-Phe present in GF-III, whereas the sequence Val-Phe was within the peptide Val-Phe-Leu-Pro-Gln in dGIF-II. Wu *et al.* (2008) isolated the ACEI peptide Phe-Leu, a sequence located in napin and cruciferin (two major canola protein components) from Alcalase hydrolyzed canola meal. This peptide sequence was located in the peptide Val-Phe-Leu-Pro-Gln of dGIF-II derived from flaxseed protein.

Table 4.21 Amino acid sequence of the peptides in the most angiotensin I-converting enzyme inhibitory-FPLC fractions of flaxseed protein digests obtained using the dynamic model

Sample	FPLC fraction	Amino acid sequence ¹	Calculated molecular mass ¹	Probability ¹
Gastric digest	GF-III	Phe-Asn-Leu-Pro-Leu-Leu (FNLPLL)	715.37	100
		Phe-Leu-Thr-Pro-Val-Phe (FLTPVF)	722.34	100
		Phe-Val-Leu-Pro-Gln-Phe (FVLPQF)	749.35	98.31
		Leu-Pro-Ala-Gly-Val-Val-Asp-Trp (LPAGVVDW)	855.38	99.97
Gastric + intestinal digest-dialysate	dGIF-II	Val-Phe-Leu-Pro-Gln (VFLPQ)	602.34	50.35
		Thr-Pro-Pro-Ala-Ala-Arg (TPPAAR)	611.34	59.05
		Trp-Asn-Leu-Asn-Ala (WNLNA)	616.30	91.31
		Leu-Leu-Val-His-Val-Val (LLVHVV)	678.44	83.58
		Asn-Leu-Asp-Thr-Asp-Leu (NLDTDL)	689.32	100
		Gln-Asn-Glu-Gly-Leu-Glu-Trp (QNEGLEW)	874.38	50.12

¹Obtained using the MassLynx software

FPLC: Fast Protein Liquid Chromatography

The single letter coding for amino acids are given within paranthesis

The amino acid sequence of SSPs in flaxseed protein is not known except for conlinin (2S; 19.06 kDa) (Truksa *et al.*, 2003). None of the bioactive peptide sequences identified in the current study was found within the primary sequence of conlinin. This indicated that conlinin may not be the precursor protein of these peptides.

Pepsin was the digestive enzyme used in the gastric phase for digestion of flaxseed proteins. All the peptide sequences of GF-III was in agreement with Nelson and Cox (2000) who reported that protein digestion by pepsin generates peptides with aromatic and hydrophobic amino acids in their C-terminal position. One peptide (Phe-Asn-Leu-Pro-Leu-Leu) had a hydrophobic amino acid in the C-terminal (Table 4.20). Three peptides (Phe-Leu-Thr-Pro-Val-Phe, Phe-Val-Leu-Pro-Gln-Phe, Leu-Pro-Ala-Gly-Val-Val-Asp-Trp) sequenced in GF-III, had aromatic amino acids in the C-terminal position.

The dialysate of gastric + intestinal digestion included absorbable peptides generated upon sequential digestion of flaxseed protein by pepsin followed by pancreatin. None of the peptides detected in dGIF-II were found to be derived from peptides in GF-III. Of the fractions of gastric digest, GF-III represented the most ACEI fraction. Therefore, the peptides in dGIF-II are probably derived from those in GF-I and GF-II or less abundant peptides in GF-III. Although not in the same order, two peptides in GF-III (Phe-Leu-Thr-Pro-Val-Phe and Phe-Val-Leu-Pro-Gln-Phe) shared four amino acids (Val, Phe, Leu and Pro) in common with the peptide Val-Phe-Leu-Pro-Gln in dGIF-II. The presence of Trp and Leu in the C-terminus of peptides Gln-Asn-Glu-Gly-Leu-Glu-Trp-Arg and Asn-Leu-Asp-Thr-Asp-Leu was according to the cleavage specificity of chymotrypsin, whereas the presence of C-terminal Arg in the peptide Thr-Pro-Pro-Ala-Ala-Arg was in accordance to the cleavage specificity of trypsin as reported by Nelson and Cox (2000). The C-terminal position of the other peptides in dGIF-II had amino acids such as Ala (Trp-Asn-Leu-Asn-Ala), Gln (Val-Phe-Leu-Pro-Gln) and Val (Leu-leu-Val-His-Val-Val). Unlike pepsin, pancreatin used in the present study was a mixture of endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (carboxypeptidases A and B). Of these, elastase cleaves bonds at aliphatic amino acid residues, whereas carboxypeptidases A and B cleave respectively aromatic amino acids

and Arg or Lys from C-terminal end of peptides or protein (Klen *et al.*, 2005). Therefore, peptides with diverse range of amino acids in the C-terminal position were expected in dGIF-II due to the simultaneous action of different proteases in pancreatin.

Cheung *et al.* (1980) highlighted the importance of the first three amino acid residues of the C-terminal part of ACEI peptide sequence in binding with ACE. It was suggested that peptide sequences containing aromatic (Trp, Tyr and Phe) or hydrophobic amino acid residues or Pro at the C-terminus or amino acids with positively charged functional groups at N-terminus are potent inhibitors of ACE (Cheung *et al.*, 1980). ACE has little affinity for substrates or competitive inhibitors with C-terminal dicarboxylic amino acids or with penultimate (next to last) Pro residues. An antepenultimate (third from last) aromatic amino acid residue enhances binding of the peptide with ACE (Cheung *et al.*, 1980). The superiority of Pro as the C-terminal residue is suggested to be due to the rigid ring structure of this amino acid that may lock the carboxyl group into a conformation favorable for interaction with the positively charged residue at the active site of the enzyme (Cushman *et al.*, 1987). Moreover, the presence of Pro residues in peptides increases their resistance to intestinal brush border enzymes and protect their degradation *in vivo* (Kim *et al.* 1972). All the identified peptides in GF-III and two peptides in dGIF-II had Pro residues within their sequences. Two of the peptides identified from GF-III (Phe-Asn-Leu-Pro-Leu-Leu and Phe-Val-Leu-Pro-Gln-Phe) had Pro in the antepenultimate position. The peptide Val-Phe-Leu-Pro-Gln identified in dGIF-II had Gln, which is the acid amide of dicarboxylic glutamic acid at the ultimate C-terminus and Pro at the penultimate position and this was in contrast with the structure activity data reported by Cheung *et al.* (1980). However, it is possible that Glu residues when present with an amino acid such as Gly, Val, Ala, Ile or Pro does not have a negative effect on the active site of ACE and can therefore be accommodated by the enzyme (Suetsuna & Chen, 2001). In agreement with Cheung (1980), C-terminal aromatic amino acids were present in three of the identified peptides in GF-III (Phe-Leu-Thr-Pro-Val-Phe, Phe-Val-Leu-Pro-Gln-Phe and Leu-Pro-Ala-Gly-Val-Val-Asp-Trp) and one peptide found in dGIF-II (Gln-Asn-Glu-Gly-Leu-Glu-Trp). Also one peptide in GF-III (Phe-Asn-Leu-Pro-Leu-Leu) and three peptides in dGIF-II (Trp-Asn-

Leu-Asn-Ala, Leu-leu-Val-His-Val-Val and Asn-Leu-Asp-Thr-Asp-Leu) had hydrophobic amino acids (Ala, Val and Leu) at the C-terminus.

Gomez-Ruiz *et al.* (2007) recently suggested that a C-terminal Leu residue may contribute significantly to ACEI potential. This is in agreement with the presence of C-terminal Leu in Phe-Asn-Leu-Pro-Leu-Leu (GF-III) and Asn-Leu-Thr-Asp-Leu (dGIF-II). The peptide Thr-Pro-Pro-Ala-Ala-Arg, present in dGIF-III had positively charged Arg residue at C-terminus. Presence of positively charged Lys or Arg at the C-terminus was previously reported in ACEI peptides such as casokinins (casein derived), bradykinin and in some synthetic inhibitors (Cheung *et al.*, 1980), which does not fit with the ACE active site model proposed by Ondetti and Cushman (1982). However, structure activity data suggest that the positive charge on the guanidine or ϵ amino group of the C-terminal Arg and Lys side chains, respectively contributes substantially to ACEI potency. For example removal of Arg from C-terminus of bradykinin results in an essentially inactive analogue (Meisel, 1993). In such peptides the mechanism of ACE inhibition includes inhibitor interaction with an anionic binding site, which is different from the catalytic site of ACE (FitzGerald & Meisel, 2000).

It is important to note that the structure activity relationship of ACEI peptides are predicted mostly based on studies of di- and tri-peptides and they may not always be extrapolated to peptides with long PCL. Wu *et al.* (2006) proposed models for food protein derived ACEI peptides of varying length based on the sequence of previously published ACEI peptides suggesting that in peptides with 4-10 amino acid residues the C-terminal tetrapeptide but not the tripeptide modulates or regulates the ACEI potency. The most preferred amino acids starting from C-terminus were Tyr, Cys for the first position, His, Trp, Met for the second position, Ile, Leu, Val, Met for the third position and Trp for the fourth position (Wu *et al.*, 2006). In agreement with Wu *et al.* (2006) peptides Leu-Pro-Ala-Gly-Val-Val-Asp-Trp (GF-III) had Val, whereas peptides Trp-Asn-Leu-Asn-Ala, Gln-Asn-Glu-Gly-Leu-Glu-Trp, and Val-Phe-Leu-Pro-Gln (dGIF-II) had Leu in the 3rd position from the C terminus. Wu *et al.* (2006) also reported that the fourth amino acid residue from C-terminus is more important in determining ACEI

potency than the second and third amino acid residues in pentapeptides and hexapeptides. None of the peptides detected in the flaxseed protein digests had Trp as the 4th residue. Based on QSAR modeling, Pripp *et al.* (2004) reported that when the PCL becomes longer the importance of C-terminal structure of peptides on ACEI potency is reduced and other steric effects, not related to C-terminal composition, begin to interfere with the ACEI potency. This may be also applicable to peptides identified in the present study as they contained relatively high PCL. Moreover, the confirmation of peptides in a specific environment is also expected to contribute to its ACEI potency (Vermeirssen *et al.*, 2004). For example the trans form of C-terminal Pro showed significant improvement in the enzyme substrate affinity rather than its cis form. (Gomez-Ruiz *et al.*, 2004). However, the structure activity relationship of ACEI peptides has not been completely elucidated.

The most important sequenced peptides are those in dGIF-II as these represent the ACEI peptides that have the potential for absorption through intestinal membrane. Vermeirssen *et al.* (2004) highlighted the importance of ACEI peptides to reach the cardiovascular system in the active form. Therefore, investigation of bioavailability of ACEI peptides derived from flaxseed protein digestion is important. The peptides detected in dGIF-II and reported in this study were more than 2-3 amino acid residues long. Several studies report the absorption of small ACEI peptides through intestine. For example, consumption of sour milk containing the ACEI tri peptides Val-Pro-Pro and Ile-Pro-Pro have been shown to lower blood pressure modestly in hypertensive subjects (Tuomilehto *et al.*, 2004). Another study showed the presence of Val-Pro-Pro in abdominal aorta of SHR suggesting that these ACEI tripeptides are absorbed in the intestine and resistant to intestinal peptidases (Masuda *et al.*, 1996). The major mechanism for absorption of Val-Pro-Pro through intestinal epithelium was found to be by paracellular diffusion (Satake *et al.*, 2002). In addition to these short chain peptides, research show the bioavailability of long chain ACEI peptides. For instance, the long chain ACEI peptides such as His-Leu-Pro-Leu-Pro generated by simulated GI digestion of human milk (Quiros *et al.*, 2008) and Ala-Leu-Pro-Met-His-Ile-Arg (lactokinin) (Vermeirssen *et al.*, 2002), are transported through the intestinal epithelium intact. In a

recent study, Platerink *et al.* (2006) reported the presence of ACEI peptides, Ile-Pro-Pro-Leu and His-Leu-Pro-Leu-Pro in human plasma upon ingestion of a drink containing them. Previous studies conducted using Caco-2 cell monolayers showed the bioavailability of antihypertensive octapeptide Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro obtained from chicken collagen hydrolysate (Shimizu *et al.*, 2010) and hexapeptide Lys-Val-Leu-Pro-Val-Pro (Sun *et al.*, 2009). The potential mechanism by which these peptides are absorbed was found to be paracellular diffusion. Similarly, in the current study, peptides that passed through the dialysis bag in the intestinal phase of the dynamic model, may represent the molecules available via passive diffusion. Due to the absence of brush border peptidases in the intestinal phase of the present study, it is difficult to conclude whether these peptides could be absorbed intact via transcellular pathway. The bioavailability of the peptide Leu-Leu-Val-His-Val-Val (dGIF-II) through transcellular pathway is questionable as peptides containing an N-terminal Leu are highly susceptible to cellular peptidase hydrolysis (Satake *et al.*, 2002).

To date, no published amino acid sequences of ACEI peptides released upon GI digestion of flaxseed protein are available, although the possibility of releasing such peptides upon enzymatic hydrolysis has been reported. The information on ACEI peptide sequences obtained in the current study will be useful in predicting the precursor SSPs of flaxseed for bioavailable ACEI peptides. Of the SSPs with known sequences, glycinin in soy, glutelin in rice, α -zein in maize, vicilin and albumin PA2 in pea, napin and cruciferin in canola have been already identified as precursor proteins generating ACEI peptides (Guang & Phillips, 2009; Hong *et al.*, 2008). Such prediction for flaxseed protein with certainty requires sequence information on the complete flaxseed proteome, which is not available at present.

4.1.6 Summary of the findings of study I

The findings of the present study fill an existing gap on the information of flaxseed, whether the GI digestion can release bioactive peptides in relation to CVD health. The ability of flaxseed protein to release ACEI and OH[•] scavenging peptides was proved using a static and a dynamic model of *in vitro* digestion. The dynamic model was

considered more physiologically relevant to the *in vivo* conditions as it included simultaneous absorption in the intestinal phase.

The DH and bioactivities of flaxseed protein digests obtained using the dynamic model and the static model of digestion are summarized in Table 4.22. The gastric + intestinal digest obtained using the dynamic model showed a significantly higher ($P<0.05$) DH than that of the static model. Moreover, differences in amino acid compositions in the gastric + intestinal digests obtained using the two models indicated that the enzyme kinetics are favored towards hydrolysis of flaxseed protein in the dynamic model than the static model to release aromatic amino acids.

Table 4.22 The degree of hydrolysis and bioactivities of flaxseed protein digests

Sample	DH (%) ¹	IC ₅₀ : ACEI activity (mg of N/mL) ¹	IC ₅₀ : OH [•] scavenging activity (mg of N/mL) ¹
Initiation of digestion	3.31 ± 0.03	N.D.	N. D.
Gastric digest	7.96 ± 0.16	0.16 ± 0.00	N. D.
Gastric + intestinal digest			
Static model	43.95 ± 0.8		0.40 ± 0.0
Dynamic model	46.78 ± 0.3		
Dialysate		0.04 ± 0.0	N. D.
Retentate		0.05 ± 0.0	N.D

¹Values are means ± standard deviations of duplicate analyses

DH: Degree of hydrolysis; ACEI: Angiotensin 1-converting enzyme inhibitory, OH[•]: Hydroxyl radical
N.D.: Not determined (due to inactivity shown at 0.67 mg/mL)

The bioactivities of gastric + intestinal digest of flaxseed protein varied with the type of digestion model used. For example, the gastric and gastric + intestinal digests of flaxseed proteins obtained using both models possessed ACEI activity. However, the ACEI activity of the gastric + intestinal digest obtained using the dynamic model was significantly higher than that from the static model.

The OH[•] scavenging activity was only shown by the gastric + intestinal digest obtained using the static model. This shows that the static model, commonly used in simulated GI digestion studies, may not correctly estimate the potential of food protein to generate bioactive peptides during GI digestion.

The flaxseed protein derived ACEI peptides possessed the potential for absorption through the small intestine by paracellular diffusion. Peptides in the molecular mass range of 0.5-1 kDa contributed mostly to the ACEI activity of the absorbable GI digest of flaxseed protein. Of the ACEI peptides generated during GI digestion of flaxseed protein, Val-Phe-Leu-Pro-Gln, Thr-Pro-Pro-Ala-Ala-Arg, Trp-Asn-Leu-Asn-Ala, Leu-Leu-Val-His-Val-Val, Asn-Leu-Asp-Thr-Asp-Leu, and Gln-Asn-Glu-Gly-Leu-Glu-Trp were found to be in the most ACEI bioavailable fraction of the GI digest.

4.1.7 Linkage to study II

As shown in study I, flaxseed protein has ACEI and OH[•] scavenging peptide sequences that can be released during digestion. Moreover, flaxseed protein has the potential of generating absorbable ACEI peptides during GI digestion. Study I was carried out using protein isolated from flaxseed.

Currently, the use of isolated flaxseed protein in food formulation is rare whereas whole flaxseed, ground flaxseed and partially defatted meal are the commonly available sources of flaxseed protein in food stores. Of these, whole and ground flaxseed are most popularly used in breads, muffins, waffles, pancakes, biscuit, crackers, bagels, tortillas, smoothies etc. Defatted flaxseed meal is also available in the market as a fiber rich source that can be used in cereals, salads, blender drinks etc. In these flaxseed protein sources, the protein component is associated with other seed matrix components, especially, mucilage and lipids. The complex seed matrix (cell walls, linked with sugars, carbohydrates and/or lipids) can influence the flaxseed protein digestibility, nature of hydrolysis products generated during GI digestion and thereby the bioactivities of the digested products. For example, the flaxseed protein source/s, which show protein digestibility similar to that of isolated flaxseed protein will indicate high

potency to generate ACEI peptides during GI digestion. Therefore, the findings of the study I cannot be used to predict the ability of the commonly available flaxseed protein sources to generate ACEI peptides upon GI digestion. Determination of any impact of seed matrix components on the digestibility of flaxseed proteins is important to identify the most suitable form of flaxseed to deliver protein derived ACEI peptides.

4.2 Study II: Effect of seed components on the digestibility of flaxseed protein

In this study, the effect of lipids and soluble polysaccharides (mucilage) of flaxseed on the digestibility of seed protein was studied using the static digestion model. The milled flaxseed products (whole, demucilaged , , demucilaged and defatted) used in the study represented mucilage, oil and protein retained in the seed matrix as they are naturally present. Comparison of protein digestibility in these materials to that of isolated flaxseed protein allowed us to understand the digestibility properties of flaxseed protein without interference of other seed components. Presence of mucilage and oil in the flaxseed protein sources could adhere to the dialysis membrane in the dynamic model interfering and underestimating the dialysis process. Therefore, the dynamic model was not used to simulate GI digestion in the present study.

4.2.1 Proximate composition of the flaxseed protein sources used for the study

The proximate composition of the different sources of laboratory prepared flaxseed protein used in the present study is given in Table 4.23. The proximate composition of milled whole flaxseed was within the range of the average composition of flaxseed reported by Bhatta (1997), except for the protein content. The protein component of the milled whole flaxseed (18.5 ± 0.3 %) was in agreement with the value (18.29 %) reported by the US Department of Agriculture, (2009) but lower than the average values (20-25 %) reported by Bhatta (1997). The composition of flaxseed varies with genetics, growing environment, seed processing and method of analysis (Pradhan *et al.*, 2010), which could be the possible reason for the observed difference. According to Hall *et al.* (2006), the protein content of flaxseed can vary between 10 % and 30 % depending on the variety and provenance of the plant. Specifically, the protein content of the seeds decrease when the oil content increases (Carter, 1993; Cunnane *et al.*, 1993).

The milled whole flaxseed had a TDF content of 27.19 ± 0.3 % comprised mostly of IDF (22.32 ± 0.7 %), whereas SDF was 4.88 ± 0.4 %. According to Madhusudhan (2009), two thirds of the TDF in flaxseed is IDF consisting of cellulose and lignin. The SDF in flaxseed is mainly comprised of mucilage (Carter, 1993).

Table 4.23 Composition of flaxseed materials (fresh weight basis) used for the simulated gastrointestinal digestion study

Sample	Moisture (%) ¹	Ash (%) ¹	Lipids (%) ¹	Protein (%) ^{1,2}	Dietary fiber (%) ¹		
					SDF	IDF	TDF
Milled whole flaxseed	5.74 ± 0.1	4.04 ± 0.0	40.62 ± 0.5	18.5 ± 0.3	4.88 ± 0.4	22.32 ± 0.7	27.19 ± 0.3
Milled and demucilaged flaxseed	0.81 ± 0.1	4.52 ± 0.1	43.93 ± 1.2	21.08 ± 0.2	3.17 ± 0.2	22.45 ± 1.7	25.63 ± 1.9
Milled, demucilaged and defatted flaxseed	4.89 ± 0.0	8.84 ± 0.1	0.23 ± 0.0	40.81 ± 0.4	2.98 ± 1.1	32.50 ± 2.4	35.06 ± 1.2
Isolated flaxseed protein	5.98 ± 0.9	4.13 ± 0.3	0.55 ± 0.1	86.01 ± 0.2	5.41 ± 0.8	2.59 ± 1.4	8.01 ± 0.6

¹Values are means ± standard deviations²% N x 6.25

SDF: Soluble dietary fibre; IDF: Insoluble dietary fibre; TDF: Total dietary fibre

The SDF in milled whole flaxseed was in agreement with previously reported values for flaxseed (5-8 %) (Cui *et al.*, 1996).

In the present study, demucilaging helped to lower SDF by 35 % and TDF by 5.7 % compared to milled whole flaxseed. Demucilaging was done with whole flaxseed; therefore soluble polysaccharides in the seed coat were removed. There are no reports on the amount of soluble polysaccharides found in the seed cotyledons and endosperm of flaxseed. Therefore, it was assumed that the seed coat mucilage was mostly removed by the method of demucilaging (using 0.5 M NaHCO₃) employed in the current study. According to Mazza and Biliaderis (1989), extraction using water at 25 °C and boiling water extracted only 3.5-5.5 % and 9 % mucilage, respectively, lower than that obtained in the current study (35 %). Removal of mucilage led to a higher percentage of protein (21.08 ± 0.2 %) and lipid (43.93 ± 1.2 %) in milled and demucilaged flaxseed compared to milled whole flaxseed (18.5 ± 0.3 % of protein and 40.62 ± 0.5 % lipid). Similar findings were reported by Alzueta *et al.* (2002).

Screw pressing of demucilaged flaxseed followed by hexane extraction dramatically decreased the oil content in the resulting milled, demucilaged and defatted flaxseed to 0.23 ± 0.0 %, and increased the protein level up to 40.81 ± 0.4 % (Table 4.23) compared to the oil (40.62 ± 0.5 %) and protein (18.5 ± 0.3 %) content in milled whole flaxseed. Oomah and Mazza (1998) also reported a 1.7 times increase in protein content of a laboratory defatted flaxseed meal compared to the original flaxseed and this corresponded to the decrease in lipid content due to defatting. The SDF % of milled, demucilaged and defatted flaxseed (2.98 ± 1.1 %) was lower than that of milled whole flaxseed (4.88 ± 0.4 %) and milled and demucilaged flaxseed (3.17 ± 0.2 %), which was expected due to the removal of oil.

The protein isolated from the milled, demucilaged and defatted flaxseed had 86.01 ± 0.2 % protein content and very low lipid content (0.55 ± 0.1 %). Although the TDF content was lower in isolated flaxseed protein, it had higher SDF content compared to the other samples (Table 4.23). The flaxseed protein used in this study was extracted by stirring

the milled, demucilaged and defatted flaxseed at an alkaline pH (8.5) followed by acid precipitation at pH 3.8. It is reported that the hemicelluloses and polysaccharides that are present as complexes with protein and polyphenol are solubilized under alkaline conditions (Selvendran, 1984). During centrifugation of the alkaline slurry of flaxseed protein extraction, any viscous mucilage remaining in the milled, demucilaged and defatted flaxseed settles between the insoluble fibrous residue and less viscous supernatant and is decanted along with the supernatant protein extract. During the acidification step the mucilage forms complexes with protein and is precipitated (Dev & Quensel, 1988). Therefore, it is hypothesized that residual mucilage remaining in the milled, demucilaged and defatted flaxseed can become soluble at alkaline extraction and co-precipitate with protein during acid precipitation leading to a relatively high SDF level in the protein isolate.

4.2.2 *In vitro* digestibility of flaxseed protein and the effects of seed components on digestibility

The effect of mucilage and flaxseed oil on the digestibility of seed protein was determined by analyzing the TCA soluble N upon digestion of flaxseed protein sources in the presence and absence of these components. As shown in Table 4.24, all flaxseed protein sources had appreciable amounts of TCA soluble N before digestion. Therefore, the net value of TCA soluble N at the end of each digestion phase was determined by deducting the % TCA soluble N at zero time from that determined at the end of each digestion phase. The net % TCA soluble N released at the gastric phase and gastric + intestinal phase during the static model of digestion were compared between different samples to investigate the effect of flaxseed matrix components on protein digestibility.

All flaxseed protein sources had limited digestibility during gastric phase (pepsin catalyzed) digestion. Milled whole flaxseed, which contained a high level of mucilage, and lipids had no or limited *in vitro* digestibility of protein (net % TCA soluble N was $0.55 \pm 1.4\%$) at the end of the gastric phase (Table 4.24). It showed a significantly lower ($P < 0.05$) protein digestibility at the end of the gastric phase compared to the other flaxseed protein sources.

Table 4.24 Digestibility of flaxseed protein in the presence of matrix components as determined using % TCA soluble N

Flaxseed protein source	TCA soluble N (%) ¹			Net TCA soluble N (%) ¹	
	Initiation of digestion	Gastric phase ²	Gastric + intestinal phase ³	Gastric phase ²	Gastric + intestinal phase ³
Milled whole flaxseed	19.62 ± 0.0	20.17 ± 1.4	32.23 ± 0.5	0.55 ± 1.4 ^c	12.61 ± 0.5 ^c
Milled and demucilaged flaxseed	9.68 ± 1.2	28.07 ± 1.4	60.68 ± 1.6	18.39 ± 1.4 ^b	51.00 ± 1.6 ^b
Milled, demucilaged and defatted flaxseed	16.06 ± 2.7	37.63 ± 2.2	82.85 ± 6.4	21.57 ± 2.2 ^b	66.79 ± 0.6 ^a
Isolated flaxseed protein	12.16 ± 0.1	40.41 ± 0.4	80.16 ± 1.8	28.25 ± 0.4 ^a	68.00 ± 1.8 ^a

¹Values are means ± standard deviations of duplicate analyses

^{abc}Means in the same column followed by the same letter are not significantly different ($P < 0.05$)

²gastric phase: 2 h

³Gastric + intestinal phase: 6 h

For each sample a sample weight containing 1g of protein was used for the simulated GI digestion

TCA: Trichloroacetic acid

There was no significant difference ($P < 0.05$) between milled and demucilaged flaxseed and milled, demucilaged and defatted flaxseed for protein digestibility during the gastric phase. This suggests that mucilage is the main seed matrix component that limits flaxseed protein digestion by pepsin. The isolated form of flaxseed protein had the highest digestibility during the gastric phase and this was significantly higher ($P < 0.05$) than the other sources.

Oomah and Mazza (1998) showed that the *in vitro* protein digestibility of flaxseed was influenced by commercial processing. The digestibility of protein in milled whole flaxseed ($0.55 \pm 1.4\%$) at the end of the gastric phase of the current simulated GI digestion study was lower than that reported by Oomah and Mazza (1998) (milled whole flaxseed: 36.1 %) upon digestion with pepsin alone. It is important to note that

these authors used 16 h of pepsin digestion, which is longer than the physiological gastric phase digestion. The *in vitro* protein digestibility of defatted flaxseed meal investigated by Oomah and Mazza (1998) upon pepsin digestion was 28.9 % whereas it was 21.57 ± 2.2 % in the present study. Unlike the milled, demucilaged and defatted flaxseed used in the present study, the flaxseed meal used by Oomah and Mazza (1998) was not demucilaged. Moreover, the meal was exposed to various heat treatments (e.g. hexane extraction of residual oil followed by further heat processing at 75 °C and 105 °C). The denaturation temperature of flaxseed protein is between 66 and 117 °C, depending on the cultivars and processing conditions (Oomah *et al.*, 2006). Therefore, in the study conducted by Oomah and Mazza (1998), the heat treatment used may have caused denaturation of flaxseed protein and facilitated enzyme accessibility, improving digestibility. This exposure of enzyme accessible sites may have overcome any negative effect caused by the residual mucilage present in the flaxseed meal on the protein digestibility.

It is also important to consider the differences in the methods used to express protein digestibility by different studies, which make comparisons difficult. For example, Oomah and Mazza (1998) determined the *in vitro* digestibility of flaxseed protein by determining the protein content in pepsin indigestible residues, whereas in the current study, the *in vitro* digestibility of flaxseed protein was calculated by expressing the TCA soluble N in the digest. Trichloroacetic acid, when used at the concentrations between 5-40 % (w/v), precipitates proteins by unfolding them and inducing a “sticky” state responsible for protein association (Sivaraman *et al.*, 1997). Therefore, the use of TCA as a precipitating agent allows separation of oligopeptides and amino acids from the undigested protein that is in soluble form. This could be another reason for the higher digestibility values reported by Oomah and Mazza (1998) compared to those obtained in the current study. Trichloroacetic acid also inactivates enzymes.

The digestibility of flaxseed protein (as shown by the net TCA soluble N %) with all seed components (milled whole flaxseed; $0.55 \pm 1.4\%$) and in isolated form (isolated flaxseed protein; 28.25 ± 0.4 %) at the end of the gastric phase was much lower

compared to the values reported for soy flour and isolated soy protein. Clatterbuck *et al.* (1980) have reported 75.3 to 84.5 % digestibility for heat treated soy flour digested with pepsin *in vitro*. Ou *et al.* (2004) and Wang *et al.* (2008) reported 86.4 % and 63.6 % digestibility for isolated soy protein after 2 h digestion with pepsin, respectively. Although the amino acid composition in flaxseed protein is comparable to that of soy (Oomah, 2001), structural features of protein and the seed matrix components associated with protein may influence digestibility. The differences in pepsin digestion pattern between flaxseed protein and soy protein could be attributed to the differences in the availability of the scissile sites of protein substrate to pepsin (Wang *et al.*, 2008). Poor solubility and presence of insoluble aggregates might result in a decrease in the availability of scissile peptide bonds (Wang *et al.*, 2008), leading to poor *in vitro* digestibility. The soluble polysaccharide (mucilage) content of soybean is negligible. Soluble polysaccharides can increase viscosity of the digesting mixture and reduce the accessibility of enzyme to the substrate protein therefore reducing protein breakdown. Moreover, the E/S ratios (1/143 w/w in Ou *et al.*, 2004 and 1/100 w/w in Wang *et al.*, 2008) used in these studies were much greater than that used in the gastric phase of the current study (1/250 w/w), which may have led to the low protein digestibility values observed in the current study.

The milled whole flaxseed (containing mucilage and lipid) had the lowest digestibility of protein (12.61 ± 0.5 %) at the end of the simulated intestinal phase and this was significantly lower ($P < 0.05$) than the other samples (Table 4.24). In spite of the limited digestibility in the gastric phase, protein digestibility was improved in milled whole flaxseed at the end of gastric + intestinal phase (12.61 ± 0.5 %), which may be due to lipid breakdown by pancreatic lipases in the presence of bile salts. This may have facilitated protein digestibility. Honig and Rackis (1979) reported 68.2 % digestibility for whole soy bean after gastric + intestinal digestion, a much higher value than the digestibility of milled whole flaxseed (12.61 ± 0.5 %) found in the current study. However, Honig and Rackis (1979) used digestion of soybeans for 18 h with pepsin and 1 h with pancreatin, which is not relevant to the physiological condition.

At the end of the gastric + intestinal phase, the protein in milled, demucilaged and defatted flaxseed showed significantly higher ($P<0.05$) digestibility (66.79 ± 0.6 %) than milled whole flaxseed (12.61 ± 0.5 %). Previously, pepsin-pancreatin digestion of defatted flaxseed meal had shown 61 % *in vitro* digestibility (Madhusudhan & Singh, 1985). In contrast to the simulated gastric phase, the protein digestibility of milled, demucilaged and defatted flaxseed was significantly higher ($P<0.05$) than that of milled and demucilaged flaxseed (51.00 ± 1.6 %) at the end of the gastric + intestinal phase. This showed that digestion of flaxseed protein by pancreatin was significantly improved when both mucilage and oil were removed than with removal of mucilage alone. In a study using soy, Honig and Rackis (1979) also showed an increased protein digestibility in dehulled defatted soy flour (81 %) compared to whole soybean (68.2 %). Of the flaxseed protein sources used, the highest *in vitro* GI digestibility was shown by isolated flaxseed protein (68.00 ± 1.8 %). Wang *et al.* (2008) determined the *in vitro* protein digestibility of flaxseed protein extract obtained from extruded flaxseed (extrusion temperature: 84-180 °C), which was in the range of 69-77 %. The high protein digestibility reported by Wang *et al.* (2008) could be possibly due to denaturation of protein during extrusion (El-Hady & Habiba, 2003).

There was no significant difference ($P<0.05$) between the protein digestibility of milled, demucilaged and defatted flaxseed and isolated flaxseed protein at the end of the intestinal phase (Table 4.24). The residual oil content in these two products was not different. Isolated flaxseed protein had slightly higher SDF content than milled, demucilaged and defatted flaxseed (Table 4.23). The remaining SDF in the isolated flaxseed protein had negligible or minimal effect on its protein digestibility (Table 4.24). The *in vitro* GI digestibility exhibited by isolated flaxseed protein (68.00 ± 1.8 %) was lower than for isolated soy protein (88.5 to 89.8 % by Clatterbuck *et al.*, 1980 and 71.04 % by Wang *et al.*, 2008). In the isolated form, the digestibility of flaxseed protein increased ~ 40 % at the end of the gastric + intestinal digestion compared to the gastric digestion. According to Wang *et al.* (2008), for soy protein, only ~7 % increase in digestion was found. Presence of trypsin inhibitors in soy protein (28-32 mg/g in soy flour and 1.2-30 mg/g in soy protein isolate; Anderson & Wolf, 1995) could be greatly

responsible for this difference. Content of trypsin inhibitors in flaxseed is very low compared to legumes (Wanasundara *et al.*, 1999).

In the current study (Study II), the digestibility of flaxseed protein was determined using the static model of digestion instead of the dynamic model. The static model and the dynamic model of GI digestion did not show notable difference in the amount of polypeptide degradation in isolated flaxseed protein in spite of the differences in bioactivities of the released peptides (Section 4.1.5.3). The findings of this study indicate that presence of both mucilage and oil in flaxseed causes low GI digestibility of flaxseed protein. To the best of my knowledge, there is no published *in vitro* study reporting the digestibility of flaxseed protein under simulated GI digestion conditions. The *in vitro* protein digestibility in extruded flaxseed reported by Wang *et al.* (2008) was upon digesting flaxseed protein extract using the multienzyme technique proposed by Hsu *et al.* (1977), which involved a multienzyme solution containing trypsin, chymotrypsin and peptidase. Although these enzymes represent those in the intestinal phase, the gastric phase digestion of protein by pepsin was omitted in their method, deviating from the actual physiological process of digestion.

Several reports indicate a reduction in protein digestibility due to the presence of lipid and fiber along with protein as shown in the present study. In a review, Wickham *et al.* (2009) highlighted the importance of incorporating surface active lipids originating from ingested food or from gut secretions in the determination of protein digestibility. These surface active lipids form complex lipid structures such as emulsions or liposomes in the gastric environment and liposomes and micellar phases in the duodenal environment, which influences protein digestibility (Wickham *et al.*, 2009). Proteins can be absorbed in the model stomach emulsion interface and can be desorbed under the duodenal conditions. Therefore, adsorption to emulsion interface makes proteins more resistant to pepsin digestion (Dufour *et al.*, 1998). Moreover, fluorescence and FTIR spectroscopy analysis has shown that proteins adsorbed to oil/water interfaces can be partly denatured. Therefore, at least part of the protein can be considered removed from the aqueous environment and therefore unavailable for digestion (Husband *et al.*, 2001).

The absorption of proteins into the emulsion created by oil of milled whole flaxseed could be responsible for the resistance shown by protein in milled whole flaxseed for gastric phase digestibility (Table 4.24). The lack of emulsion formation could be the reason for higher gastric phase protein digestibility in protein isolate compared to other protein sources. Similarly, the defatting of demucilaged flaxseed also resulted in an improvement in protein digestibility although it was not significantly different ($P<0.05$) than that of milled and demucilaged flaxseed.

In addition to oil, consumption of food with high fibre content, mainly in soluble form decrease the digestibility and availability of food nutrients including proteins. The susceptibility of β lactoglobulin to proteolysis by trypsin and chymotrypsin was retarded by soluble polysaccharides such as pectin, gum arabic and xylan (Mouecoucou *et al.*, 2004a). A significant reduction in the *in vitro* GI digestibility of peanut protein isolate was also reported in the presence of gum arabic and xylan (Mouecoucou *et al.*, 2004b). The authors hypothesized that this effect was due to existence of nonspecific interactions between molecular species in protein-polysaccharide mixtures. Some fibre types are reported to reduce the activity of enzymes involved in protein digestion while others have no effect. For example, Schneeman (1978) showed a decrease in chymotrypsin activity with the incorporation of xylan, whereas pectin improved the activity of trypsin and chymotrypsin. Schneeman (1978) reported that some enzymes in the *in vitro* system may be absorbed into the fibre matrix and thereby lose activity due to reduced availability. Under *in vivo* conditions, increased viscosity of digesta in the small intestine caused by soluble fibres will reduce the gut transit time due to suppressed intestinal contractions, which lead to less mixing of dietary components with digestive enzymes (Degen *et al.*, 2007). In a study carried out using broiler chicks, Rebole *et al.* (2002) identified that when mucilaginous material extracted from flaxseed was added to maize /soybean meal based diets, the mucilage had a negative effect on lipid digestibility and had no effect on protein digestibility. In contrast, Alzueta *et al.* (2001) found a decrease in amino acid digestibility of broiler chicks fed with flaxseed. The increase in viscosity of digesta due to the presence of mucilage in flaxseeds has been highlighted as the reason for reduced amino acid digestibility. The conditions used

in the present *in vitro* study closely resemble the human digestive system and therefore a similar effect of flaxseed mucilage could be expected when consumed by humans.

The digestibility of flaxseed protein was compared with that of casein under the static model of digestion. Protein in milled whole flaxseed and milled and demucilaged flaxseed had lower digestibility than casein ($65.46 \pm 1.7\%$; Section 4.1.5.1). Under the simulated GI digestion conditions used, the digestibility of milled, demucilaged and defatted flaxseed as well as isolated flaxseed protein were similar to that of casein. Casein, due to its low disulfide bond content and high Pro content, has the configuration of a non-rigid protein molecule. Action of pepsin can destabilize the micellar structure of casein and open more sites for enzymatic action. Plant proteins such as soybean has lower digestibility than casein due to the more complex and chemically stable structure of soy protein (Baglieri *et al.*, 1995). Two major storage proteins have been identified in flaxseeds; a predominant salt soluble fraction with high MW (11-12S), and a water soluble basic component with low MW (1.6-2S) (Chung *et al.*, 2005; Madhusudhan & Singh, 1983, 1985). Similarities between the properties of the major storage protein of flaxseeds and those of other important oilseeds have been suggested (Oomah & Mazza, 1993). However, Chung *et al.* (2005) reported that flaxseed protein has low sulfhydryl (S-H) content and high disulfide (S-S) content. The values for S-S and S-H content of 11-12S flaxseed protein are lower than that of soy 11S protein. There is a possibility that digestibility of flaxseed protein can be greater than that of soy protein. However, most of the properties of flaxseed protein are still awaiting investigation. Therefore, the exact reason why flaxseed protein show similar digestibility to casein is under explained.

4.2.3 Degradation of flaxseed proteins during gastrointestinal digestion: Effect of matrix components

Figure 4.17 shows the polypeptide bands remained in the undigested residue following digestion of flaxseed protein sources at the gastric phase and the gastric + intestinal phase compared to the samples taken at the initiation of digestion (zero time).

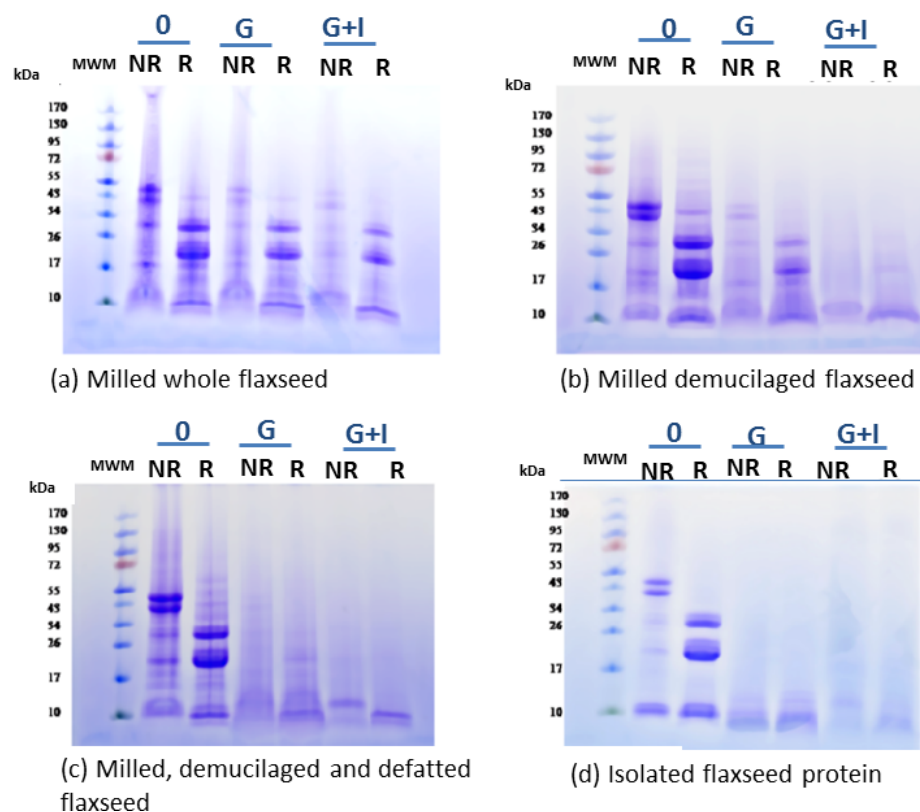


Figure 4.17 Degradation pattern of flaxseed protein in the presence and absence of matrix components (0=undigested protein; G= gastric digested; G+I= gastric + intestinal digested; NR=non reducing; R=reducing; MWM: Molecular weight markers). Gels used were gradient mini gels (resolving 8-25% T and 2% C, stacking zone 4.5 %T and 3 %C, 43×50×0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4). Each well was loaded with 5 µg protein

Figure 4.17 further shows the degradation pattern of flaxseed protein from different sources during GI digestion *in vitro*. A clear difference was observed in the protein digestion pattern between different samples. Under the NR condition, five major polypeptide bands (~48, 41, 29, 20 and 13 kDa) were visible in all forms of undigested flaxseed protein along with a few minor bands. The band intensities varied depending on the type of protein source used. The polypeptide bands of 48 kDa and 41 kDa were the most intensely stained. Similar results were reported by Chung *et al.* (2005) for the major fraction of flaxseed protein.

Under reducing conditions, the 48 kDa and 41 kDa disappeared and several intense bands having molecular weight of 34-26 kDa, 26-17 kDa and ~ 10 kDa were observed in the undigested samples (Figure 4.17) indicating the presence of S-S bonded 41 and 48 kDa peptide oligomers. Most of the 12S protein that have S-S bonded subunits are reported to dissociate into constituting acidic and basic polypeptides with molecular weights in the range of 30-39 kDa and 20-27 kDa, respectively (Marcone *et al.*, 1998). There were no bands appearing below ~13 kDa under reducing conditions, which indicated that the ~13 kDa polypeptide was free from S-S bonds. The presence of S-S bonded oligomers in flaxseed protein was previously reported (Chung *et al.*, 2005; Sammour, 1988). The major fraction of flaxseed protein was reported to contain 61.4 μ mol of S-S bonds per gram of protein (Chung, 2001). According to Sammour (1988) the flaxseed protein extracted with Tris/HCl buffer exhibited five S-S bonded bands (56, 52, 51, 49 and 48 kDa) under NR conditions, which on reduction gave acidic subunits (~35 kDa) and basic subunits (21-15 kDa).

Krause *et al.* (2002) showed that the four major bands (55, 50, 46 and 36 kDa) detected in flaxseed protein extracted with NaCl corresponded to 11S globulin and the faintly stained polypeptide bands (54, 36 and 21 kDa from 7S globulins and 7-10 kDa) were free from interchain S-S bonds. Sammour (1999) extracted protein from flaxseed flour with water, buffer, urea and SDS and observed that all the extracts contained six major bands with MWs of 55 kDa, 50 kDa, 47 kDa, 45 kDa, 43 kDa and 41 kDa under NR conditions. The intensely stained protein bands in milled whole flaxseed observed in our study were within this MW range. Chung *et al.* (2005) identified that the major

fraction of flaxseed protein had two prominent polypeptide bands (40 and 48 kDa), which resulted in three predominant bands (20, 23 and 31 kDa) under reducing conditions. Oomah and Mazza (1998) subjected flaxseed protein extracted with NaCl to SDS-PAGE under reducing conditions and observed four polypeptide bands with approximate molecular weight of 14, 24, 25 and 34 kDa. The observed differences in molecular mass of polypeptide bands resulted in the present study compared to other studies could be due to the differences in protein extraction medium and the cultivar used. A previous study has shown that the molecular weight of polypeptide bands observed in SDS-PAGE can vary with the type of reagent used for protein extraction (Marfo & Oke, 1989).

The degradation pattern of flaxseed protein bands during each phase of digestion was determined when unhydrolyzed protein and the remaining residues upon GI digestion were separated in SDS-PAGE (Figure 4.18; Table 4.25). Initially, seven protein bands were detected in the milled whole flaxseed (Figures 4.17 and 4.18 (I) b). Following the simulated gastric + intestinal digestion of milled whole flaxseed, all these bands lost their intensity compared to the sample at the initiation of digestion (Figures 4.17 and 4.18 (I) d; Table 4.25). Gastric digestion contributed to higher level of degradation of protein bands in milled whole flaxseed flour compared to the intestinal digestion. The protein bands of ~29 kDa and ~18 kDa which appeared as minor bands were the ones that were completely digested during simulated GI digestion (Table 4.25). However, no complete disappearance of any of the major polypeptide bands was observed. Of these, the bands at ~20 and ~13 kDa were found to be the most resistant to digestion with only 20 % loss in intensity (Table 4.25).

A notable decrease in the intensity of protein bands was detected in milled whole flaxseed at the end of digestion phases (Figure 4.18(I); Table 4.25), which was in contrast to the digestibility values determined as % TCA soluble N (Table 4.24).

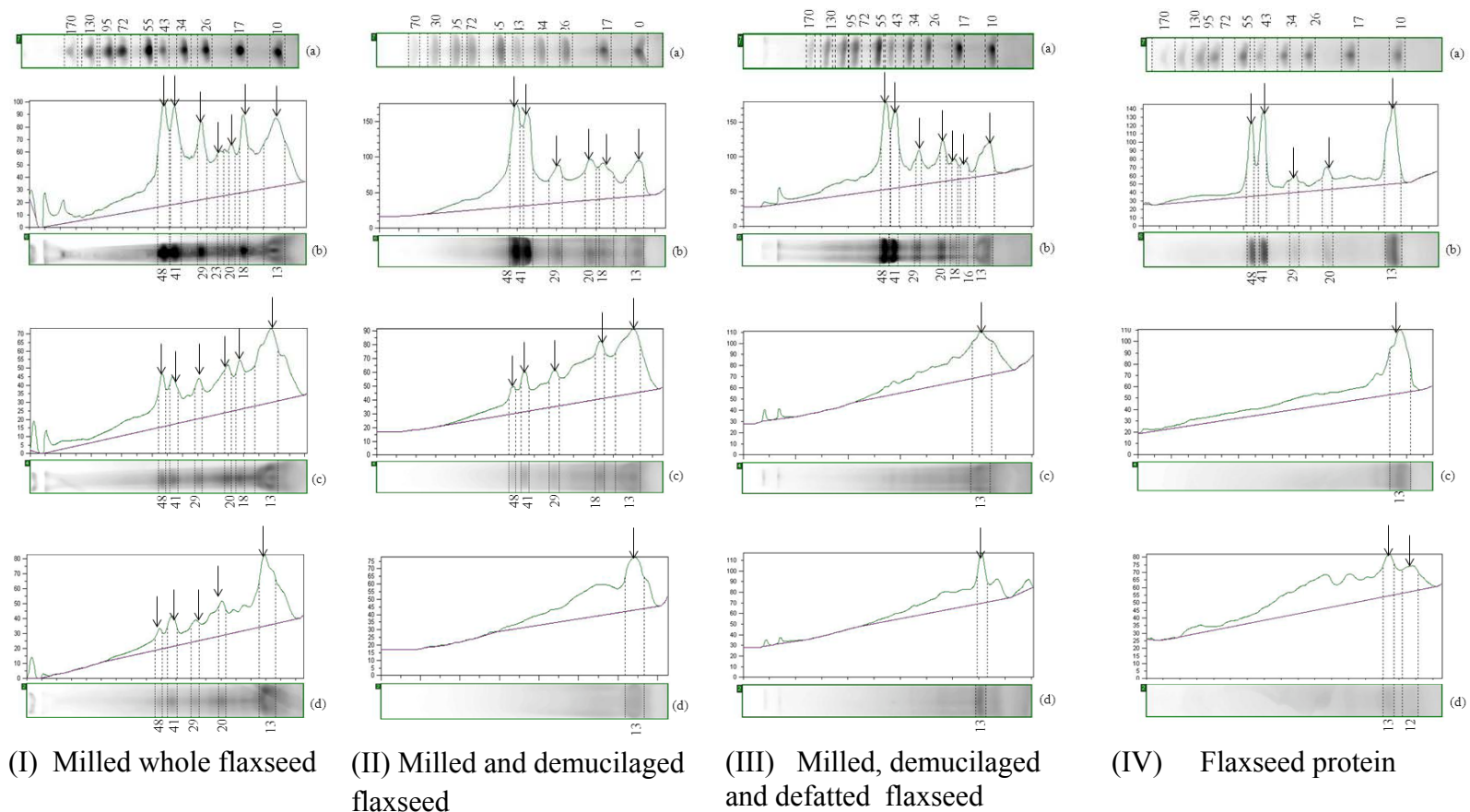


Figure 4.18 Electropherogram images (X axis: Pixel position; Y axis: Pixel intensity) of polypeptide bands in the undigested protein residue of flaxseed protein sources (non-reducing) at the end of each digestion phase. (a) Molecular weight markers (kDa), (b) Initiation of digestion, (c) at the end of gastric phase (2 h), (d) at the end of gastric + intestinal phase (6 h). Peaks designated with arrows were followed for intensity analysis

Table 4.25 Difference (%) in polypeptide band intensity in flaxseed protein residues at the end of digestion phases

Source	Digestion phase	Percentage difference of pixel intensity ¹ of polypeptide bands							
		48 kDa	41 kDa	29 kDa	23 kDa	20 kDa	18 kDa	16 kDa	13 kDa
Milled whole flaxseed	Gastric (2 h)	-71	-67	-63	-100	-18	-42	N.D.	-34
	Gastric + intestinal (6 h)	-86	-70	-72	-100	-20	-100	N.D.	-19
Milled and demucilaged flaxseed	Gastric (2 h)	-89	-79	-54	N.D.	-100	-35	N.D.	+58
	Gastric + intestinal (6 h)	-100	-100	-100	N.D.	-100	-100	N.D.	-12
Milled, demucilaged and defatted flaxseed	Gastric (2 h)	-100	-100	-100	N.D.	-100	-100	-100	+3
	Gastric + intestinal (6 h)	-100	-100	-100	N.D.	-100	-100	-100	-51
Isolated flaxseed protein	Gastric (2 h)	-100	-100	-100	N.D.	-100	N.D.	N.D.	+15
	Gastric + intestinal (6 h)	-100	-100	-100	N.D.	-100	N.D.	N.D.	-81

¹Negative and positive values indicate reduction and gain, respectively of pixel intensity of polypeptide bands compared to the initiation of digestion (e.g. -100 indicates a complete loss of a polypeptide band)

N.D.: Not detected

Band molecular masses refer to those in Figure 4.18

After each phase of digestion, the pH of the digest was reduced to 3.8 (pH at which flaxseed protein is minimally solubilized) prior to the recovery of the undigested residue used for SDS-PAGE. Our previous studies indicated that at least 10 % of flaxseed proteins are soluble at this pH (Section 4.1.2). Therefore, even though there was limited protein digestibility in milled whole flaxseed during the gastric phase, the solubility of proteins at pH 3.8 could be the reason for the significant reduction of intensity of polypeptide bands.

The protein degradation pattern of milled and demucilaged flaxseed showed a notable difference with greater polypeptide band degradation when compared to that of milled whole flaxseed (Figures 4.17 and 4.18; Table 4.25). In contrast to the seven protein bands detected in the milled whole flaxseed, only six bands were detected in the milled and demucilaged flaxseed at the initiation of digestion (Figure 4.18(II) b). The minor protein band, which was around 23 kDa, was not visible in the electrophoregram of the milled and demucilaged flaxseed (Figure 4.18(II) b). However, similar to milled whole flaxseed, five major polypeptide bands were detected in the milled and demucilaged flaxseed. At the end of the gastric phase digestion, there was an increase in the intensity of the protein band at ~13 kDa (Table 4.25). This could be a result of generation of new oligopeptides around 13 kDa, which are insoluble at pH 3.8, during gastric digestion. After the simulated intestinal digestion phase, the majority of the polypeptides in the milled and demucilaged flaxseed were completely hydrolyzed except for the polypeptide ~13 kDa, which resisted digestion showing only 12 % reduction of the pixel intensity (Figure 4.18(II) d; Table 4.25).

In milled, demucilaged and defatted flaxseed, seven polypeptide bands were detected at the initiation of digestion (Figure 4.18(III) b). Instead of the polypeptide band around 23 kDa, which was observed in the milled whole flaxseed, there was the appearance of a new minor band of ~16 kDa (Figure 4.18(III) b). It was not certain whether these minor bands are true new proteins or indicate contamination. The loss of intensity of polypeptides bands at the end of gastric phase and small intestinal phase was greater in milled, demucilaged and defatted flaxseed than that of oil containing but mucilage free

protein source (milled and demucilaged flaxseed). A complete loss of intensity of the polypeptide bands including the intensely stained bands of 48 and 41 kDa at the end of gastric phase was observed for the oil and mucilage free flaxseed protein source. Similar to milled and demucilaged flaxseed, the existence of a LMW polypeptide band (~13 kDa) was visible at the end of gastric + intestinal phase (NR). The intensity of this band was 2 % greater at the end of gastric phase digestion than that detected at the initiation of digestion indicating the generation of a digestion resistant oligopeptide of ~13 kDa during gastric digestion. This band still existed at the end of gastric + intestinal digestion, at 51 % lower intensity than that of initiation of digestion showing its digestion resistance (Figure 4.14(III) d; Table 4.25).

The isolated flaxseed protein showed five polypeptide bands (Figures 4.17d and 4.18(IV) b). The minor bands of ~23 kDa, 18 kDa and 16 kDa that were present in other flaxseed protein sources were not detected with appropriate intensity in the isolated protein. Certain minor protein bands may have been solubilized at pH 3.8 and removed following centrifugation, which was used for the initial isolation of flaxseed protein and this could be the reason for not detecting them. Similar to milled, demucilaged and defatted flaxseed (free of oil and mucilage), in the isolated protein (free of cell wall components, oil and mucilage), the majority of the protein bands including ~48 and 41 kDa were completely absent at the end of both digestion phases indicating high susceptibility of these bands for digestion by pepsin and pancreatin. Also, the residue of gastric digestion of isolated protein showed increased pixel intensity of the ~13 kDa band (Figure 4.18(IV) c; Table 4.25), probably due to the generation of new undigestible oligopeptides during gastric phase digestion.

The residue of gastric + intestinal digestion of isolated protein also had the ~13 kDa band that was stained with lower intensity (81 % compared to initiation of digestion) than that of milled, demucilaged and defatted flaxseed (51 %; Table 4.25). Moreover, at the end of gastric + intestinal phase a new band appeared below 13 kDa, which can be considered as a degraded product of the ~13 kDa band. (Figure 4.18(IV) d).

The polypeptide degradation pattern of milled and demucilaged flaxseed, milled, demucilaged and defatted flaxseed and isolated protein were in agreement with the digestibility values obtained for these samples at different digestion phases (Table 4.25). The results showed that the hydrolysis of all the polypeptides in flaxseed protein was limited in the presence of mucilage and oil. They were susceptible to hydrolysis by the digestive enzymes when oil and/or mucilage were removed. All the forms of flaxseed protein used for the digestion study had a low molecular weight band (~13 kDa) that resisted digestion by both pepsin and pancreatin.

4.2.4 Summary of the findings of study II

The *in vitro* GI digestibility of protein in different flaxseed protein sources when arranged according to the descending order was as follows; isolated flaxseed protein and milled, demucilaged and defatted flaxseed > milled and demucilaged flaxseed > milled whole flaxseed. Presence of both mucilage and oil limits protein digestibility of flaxseed protein. According to the results, the most suitable form of flaxseed that can be consumed to obtain the benefit of flaxseed protein derived bioactive peptides will be the isolated protein or the milled, demucilaged and defatted flaxseed.

At present no *in vitro* simulated GI digestion studies have been published on the effect of flaxseed mucilage and oil on flaxseed protein digestibility. Knowledge of the influence of these matrix components provides information on the most suitable form of flaxseed consumption. The physical form in which foods are consumed is an important factor to be considered in determining the bioavailability of nutrients (Bjorck *et al.* 1994). The measure of peptides and amino acids released during proteolysis indicates how proteins can be broken down into small molecules that are more easily absorbed and utilized (Restani *et al.*, 1992). Therefore, the digestibility of flaxseed protein is an indication of its efficacy to generate bioactive peptides during GI digestion. The *in vitro* study discussed under study I indicated that flaxseed protein digestion can generate potentially bioavailable ACEI peptides. Consumption of flaxseed in the form of whole seed or milled whole flaxseed may limit the generation of such bioactive peptides due to limited digestibility. Therefore, mucilage and oil removed ground flaxseed or protein

free of oil, mucilage and cell wall components would be ideal sources to release ACEI peptides during GI digestion.

4.2.5. Linkage to study III

Findings of study II showed that the removal of mucilage and oil, which are the two biopolymers available in large amounts in flaxseed, is necessary to enhance protein digestibility. Therefore, removal of mucilage and oil in flaxseed could improve the ability of flaxseed protein to release ACEI peptides of interest. These findings can be utilized to generate flaxseed protein products as a source of bioactive peptides, as well as to extend flaxseed value chain through protein product development. Flaxseed, which is gaining popularity as a functional food is mainly consumed as whole seed either as it is or in milled form. The low digestibility of milled whole flaxseed indicated the limited ability of obtaining the advantage of flaxseed protein derived ACEI peptide if consumed. Use of isolated protein or ground form of oil and mucilage-free flaxseed in bioproducts as well as generation of flaxseed protein derived bioactive peptide rich products will be important choices to deliver the flaxseed protein derived ACEI peptides to consumers. Since flaxseed protein has limited food applications, conversion of flaxseed protein into a hydrolysate containing bioactive peptides will be ideal in this regard. The limited hydrolysis of flaxseed proteins using commercially available food grade proteolytic enzymes will produce hydrolysates possessing a range of bioactivities as well as enhanced functionalities that will find application in wide variety of functional food products. Therefore, the potential for obtaining bioactive peptides from flaxseed protein using enzymatic means other than GI enzymes will be investigated in the next study and in order to explore ways to develop flaxseed protein bioproducts.

4.3 Study III: Generation and characterization of bioactive peptides by exogenous hydrolysis of flaxseed proteins with a commercial fungal protease

This study was aimed at identifying the potential of generating bioactive protein hydrolysates for functional food applications via hydrolysis of flaxseed protein with Flavourzyme® which is a commercially available food grade protease. The resulting flaxseed protein hydrolysates (FPHs) were screened for several cardioprotective bioactivities namely ACEI activity, bile acid binding ability, antioxidant activities (OH• and O₂^{•-} scavenging activities, ability to inhibit linoleic acid oxidation, metal chelating activity) and antithrombotic activity. The effect of E/S (Leucine aminopeptidase units/g protein) and hydrolysis time (h) on the DH and bioactivities of FPHs were also determined. Of the bioactivities screened, ACEI and OH• scavenging activities investigated in study I, were selected for further analysis of bioactive peptides in the FPHs.

4.3.1 Hydrolysis of flaxseed protein by Flavourzyme

The isolated flaxseed protein was hydrolyzed by Flavourzyme at different E/S and time of hydrolysis. The enzymatic hydrolysis of flaxseed protein was carried out at the optimum temperature (50 °C) for the activity of Flavourzyme. Since Flavourzyme has its optimum activity in a range of pH (5-9), the pH of the reaction mixture was adjusted to pH 7 prior to hydrolysis in order to provide optimal conditions for enzyme action. During hydrolysis pH of the mixture decreased from 7 to 5 (approximately). However, the pH value remained within the optimum range throughout the hydrolysis.

Flavourzyme is a commercially available fungal protease, extracted from *Aspergillus oryzae*. It is also known as leucyl aminopeptidase consisting of exopeptidase and endoprotease complex, which can be used for extensive hydrolysis of food proteins (Hamada, 2000; Novozyme, 2010). Protein hydrolysates with bioactive peptides are important ingredients in functional food applications. However, the free amino acids and small peptides in protein hydrolysates also act as taste compounds themselves or serve as reactants for the Maillard reaction. Therefore, such hydrolysates contribute to taste and flavor of the end product (Lin *et al.*, 2008). Hydrolysis of proteins by proteases can

cause undesirable bitter flavors due to the formation of polypeptides of a certain length with hydrophobic peptides at the end of the polypeptide chain (Hamada, 2000). This bitterness can limit the application of the resulting hydrolysate as an ingredient in food applications. Flavourzyme can cleave off the hydrophobic residues in proteins and avoid bitter flavors in resulting hydrolysates (Hamada, 2000). Moreover, Flavourzyme generates short peptides upon protein hydrolysis, which are less resistant to further hydrolysis by proteolytic enzymes. Therefore, Flavourzyme was preferred in this study over the other commercially available fungal proteases, which are mainly endoproteases.

The lyophilized FPHs had a N content ranging from 11.18 % to 12.51 %. Table 4.26 shows the DH and average PCL of the resulting FPHs. Flavourzyme caused extensive hydrolysis (>50 % DH) of flaxseed protein under a majority of the E/S and hydrolysis time combinations used (Table 4.26). The DH, which is a measure of the number of peptide bonds cleaved as a percentage of total peptide bonds in the protein (Adler-Nissen, 1986), ranged from 11.94 ± 4.1 % to 70.62 ± 5.9 % (Table 4.26) for flaxseed protein and increased with the increasing E/S and time of hydrolysis. The maximum DH (70.62 ± 5.9 %; PCL: 1.4) was achieved when the protein was hydrolyzed at an E/S of 80 (LAPU/g of protein) for 20 h. The lowest DH (11.94 ± 4.1 %; PCL: 9.2) was when protein was hydrolyzed at an E/S of 1.5 (LAPU/g of protein) for 12 h. The average PCL of the FPHs ranged from 1.4 to 9.2 and decreased with increasing DH (Table 4.26). Average PCL of most hydrolysates was <4 indicating extensive hydrolysis of flaxseed protein have occurred. Therefore, the majority of the FPHs were expected to contain predominantly short chain peptides. Results showed that both the time of hydrolysis and E/S had significant effects ($P<0.01$) on DH values, whereas only the E/S had a significant effect ($P<0.01$) on PCL.

The high DH observed in the current study was as expected due to the exopeptidase and endoprotease activity of Flavourzyme. However, Pedroche *et al.* (2002) highlighted the necessity of using more than one protease to obtain extensive DH (>50 %) stating that one enzyme cannot achieve such a high DH within a reasonable time. However, in the

current study, flaxseed protein isolate was identified as a potential substrate protein for Flavourzyme to generate hydrolysates with over 50 % DH.

Table 4.26 Hydrolysis conditions employed and degree of hydrolysis and peptide chain length resulted in for the flaxseed protein hydrolysates

E/S (LAPU/g protein) ¹	Time of hydrolysis (h) ¹	DH (%) ²	Average PCL ²
1.5	12.0	11.94 ± 4.1 ^c	9.16 ± 3.2
15.0	4.0	25.13 ± 1.6 ^c	3.99 ± 0.3
15.0	20.0	47.30 ± 3.5 ^b	2.12 ± 0.2
47.5	0.7	24.63 ± 2.9 ^c	4.11 ± 0.5
47.5	12.0	64.24 ± 0.7 ^a	1.56 ± 0.02
47.5	23.3	65.42 ± 5.1 ^a	1.54 ± 0.1
80.0	4.0	57.15 ± 4.1 ^{ab}	1.76 ± 0.1
80.0	20.0	70.62 ± 5.9 ^a	1.42 ± 0.1
93.5	12.0	64.86 ± 4.7 ^a	1.55 ± 0.1

¹ Experimental points obtained using a Central Composite Rotatable Design; Temperature: 50°C

² Values are means ± standard deviations of duplicate analyses

^{abc} Means in the same column followed by same letters are not significantly different ($P < 0.05$)

E/S: Enzyme to substrate ratio; LAPU: Leucine amino peptidase unit; DH: Degree of hydrolysis; PCL: Peptide chain length

Hydrolysis for 12 h and 23.3 h at an E/S of 47.5 LAPU/ g protein, 4 h and 20 h at an E/S of 80 LAPU/ g protein and 12 h at 93.5 LAPU/ g protein generated hydrolysates with DH above 50 % (Table 4.26). A hydrolysate with high DH is expected to be enriched with short chain peptides and amino acids released from the parent protein. Studies have shown the rapid absorption of short chain peptides such as di and tri peptides by the

human intestine (Adibi & Morse, 1971). Therefore, protein hydrolysates with short peptides and bioactive properties will be beneficially desirable ingredients in the development of physiologically functional foods.

The maximum DH exhibited by flaxseed protein in the current study ($70.62 \pm 5.9\%$) was greater than the DH values reported for Flavourzyme hydrolyzed proteins from other oilseeds such as soy, which had DH of 39.5 % upon 8 h of hydrolysis (Hrckova *et al.*, 2002) and DH of 5.46 to 17.86 % upon 1 to 8 h of hydrolysis (Tsou *et al.*, 2010) and sunflower that had DH of 42.2 % upon 3 h of hydrolysis (Villanueva *et al.*, 1999). The maximum DH value ($70.62 \pm 5.9\%$) of FPHs obtained by Flavourzyme hydrolysis alone was also greater than the recently reported values for Alcalase-Flavourzyme hydrolysates of yellow pea protein hydrolysate (DH: 58.89 %) but lower than the Kabuli (DH: 77.58 %) and Desi (DH: 77.53 %) chickpea protein hydrolysates prepared by the same enzymes (Barbana & Boye, 2010). Alcalase, unlike Flavourzyme, is an endoprotease with very high substrate specificity and it can hydrolyze most of the peptide bonds within a protein molecule (Zhang *et al.*, 1998). Generally, alkaline proteases, including Alcalase, exhibit higher activities than do acid or neutral proteases such as Flavourzyme (Rebeca *et al.*, 1991).

Pre-digestion with Alcalase, exposes a number of N terminal sites, facilitating hydrolysis by Flavourzyme (Pedroche *et al.*, 2002). Therefore, a hydrolysate prepared by sequential hydrolysis of Alcalase and Flavourzyme tend to have higher DH than when Flavourzyme was used alone. However, the variations in DH could be due to the differences in the protein composition, time of hydrolysis and E/S levels employed and differences in sensitivity of the methods used for determining DH.

4.3.2 Bioactivities of flaxseed protein hydrolysates

The freeze-dried, FPHs were screened for several *in vitro* bioactivities and the results are shown in Table 4.27.

Table 4.27 Angiotensin I-converting enzyme inhibitory, antioxidant and antithrombotic activities of flaxseed protein hydrolysates

E/S (LAPU/g protein) ¹	Time of hydrolysis (h) ¹	ACEI activity (%) ^{2,3}	Antioxidant activities (%) ¹				Antithrombotic activity (%) ^{2,3}
			OH [•] scavenging activity ^{2,4}	O ₂ ^{•-} scavenging activity ^{2,3}	Inhibition of linoleic acid oxidation ^{2,3}	Metal chelating activity ^{2,3}	
1.5	12.0	88.29 ± 1.1 ^a	21.63 ± 0.6 ^a	37.32 ± 1.1 ^a	94.33 ± 4.5 ^a	15.95 ± 0.7 ^{cd}	8.39 ± 1.9
15.0	4.0	87.83 ± 1.3 ^a	21.46 ± 0.5 ^a	29.39 ± 1.9 ^{de}	71.48 ± 3.9 ^b	24.91 ± 3.1 ^{ab}	0
15.0	20.0	78.96 ± 0.1 ^b	19.68 ± 1.3 ^{ab}	35.84 ± 2.6 ^{ab}	49.23 ± 0.3 ^{cd}	20.22 ± 1.12 ^{bc}	0
47.5	0.7	86.45 ± 0.4 ^a	22.08 ± 0.3 ^a	26.33 ± 2.5 ^c	66.81 ± 8.9 ^b	16.57 ± 0.4 ^{cd}	19.83 ± 2.1
47.5	12.0	76.06 ± 0.8 ^c	17.44 ± 1.3 ^{abc}	36.59 ± 2.1 ^{ab}	69.80 ± 3.3 ^b	17.62 ± 2.7 ^{cd}	0
47.5	23.3	73.19 ± 0.5 ^d	16.53 ± 3.5 ^{abc}	36.31 ± 0.7 ^{ab}	62.14 ± 7.9 ^{bc}	14.40 ± 1.2 ^d	10.65 ± 3.2
80.0	4.0	76.76 ± 0.2 ^{bc}	18.33 ± 2.3 ^{abc}	33.37 ± 1.0 ^{bcd}	35.28 ± 5.5 ^d	21.22 ± 1.4 ^{abc}	0
80.0	20.0	71.59 ± 0.4 ^d	12.48 ± 2.7 ^c	29.80 ± 1.2 ^{cde}	0.71 ± 4.7 ^e	26.08 ± 2.1 ^a	0
93.5	12.0	72.65 ± 0.8 ^d	14.60 ± 0.8 ^c	39.41 ± 1.9 ^a	88.17 ± 1.4 ^a	23.46 ± 0.7 ^{ab}	11.34 ± 0.3
Unhydrolyzed protein		0	0	0	19.62 ± 1.6	29.57 ± 0.7	0
Bioactivity of standards		Captopril	Carnosine	Ascorbic acid	Ascorbic acid	Carnosine	Antithrombin
		100 ± 0.0	63.50 ± 4.0	62.24 ± 1.1	63.87 ± 0.5	84.99 ± 0.4	100.00 ± 0.0

¹Experimental points obtained using a Centre Composite Rotatable Design; Temperature was maintained at 50°C ; ²Values are means ± standard deviations of duplicate analyses; ^{abcde} Means in the same column followed by the same letter are not significantly different ($P < 0.05$); ³Concentration: 0.67 mg/mL; ⁴Concentration: 0.5 mg/mL; E/S: Enzyme to substrate ratio; LAPU: Leucine amino peptidase units; ACEI: Angiotensin 1-converting enzyme inhibitory; OH[•]: Hydroxyl radical; O₂^{•-}: Superoxide radical; Unhydrolyzed protein was not included in statistical analysis

4.3.2.1 Angiotensin I-converting enzyme inhibitory activity

The ACEI activity of the FPHs (0.67 mg hydrolysate/mL in the assay mix) ranged from $71.59 \pm 0.4\%$ to $88.29 \pm 1.1\%$ (Table 4.27) indicating that all the FPHs had ACEI activity over 70 %. Maximum ACEI activity ($88.29 \pm 1.1\%$) was observed when the protein was hydrolyzed for 12 h at E/S of 1.5 LAPU/g protein. The two hydrolysates prepared using E/S (LAPU/ g protein) of 15 and 47. 5 for 4 h and 0.7 h, respectively also had ACEI activities above 85 % at the concentrations used. The ACEI activity of these three FPHs were significantly greater ($P<0.05$) compared to the others.

Hydrolysis of proteins with heat, alkali and acids also can generate bioactive peptides. Therefore, the ACEI activity was also determined for the unhydrolyzed protein and for the protein treated with exact time and temperature combinations as used in the experiment with no added Flavourzyme. These test samples including unhydrolyzed protein showed no ACE inhibition. This confirms that the ACEI activity of the FPHs is due to the peptides released as a result of Flavourzyme catalyzed hydrolysis. Captopril, which was used as the standard ACE inhibitor showed 100 % inhibition of ACE at 0.67 mg/mL (Table 4.27).

The ACEI activities of the FPHs showed a significant decrease ($P<0.05$) when DH was beyond 25 % (Figure 4.19), indicating that extensive hydrolysis resulted in low PCL and reduced ACEI activity. According to the results, high molecular weight (HMW) peptides in the FPHs were better ACE inhibitors than the low molecular weight (LMW) peptides. Contrasting results were shown for Flavourzyme hydrolysates of tilapia fish protein (Raghavan & Kristinsson, 2009) and soy protein (Chiang *et al.*, 2006), with these studies reporting an increased ACEI activity with increase in DH. However, the hydrolysates in these studies, which had been tested for ACEI activity, had lower ranges of DH values. The maximum DH values in both studies (25 % or less) were lower compared to the current study (70.62 %). Since unhydrolyzed flaxseed protein did not show any ACEI activity, with the increase in DH, the ACEI value would reach a maximum and then start to decrease thereafter. For example, for Alcalase hydrolyzed chickpea protein, Pedroche *et al.* (2002) observed an increase in ACEI activity up to 30

% DH and reported a decrease in ACEI activity thereafter. The lowest DH observed in our study was 11.94 ± 4.1 % (Table 4.26). Hence the response of ACEI activity in relation to DH below 11.94 % could not be detected.

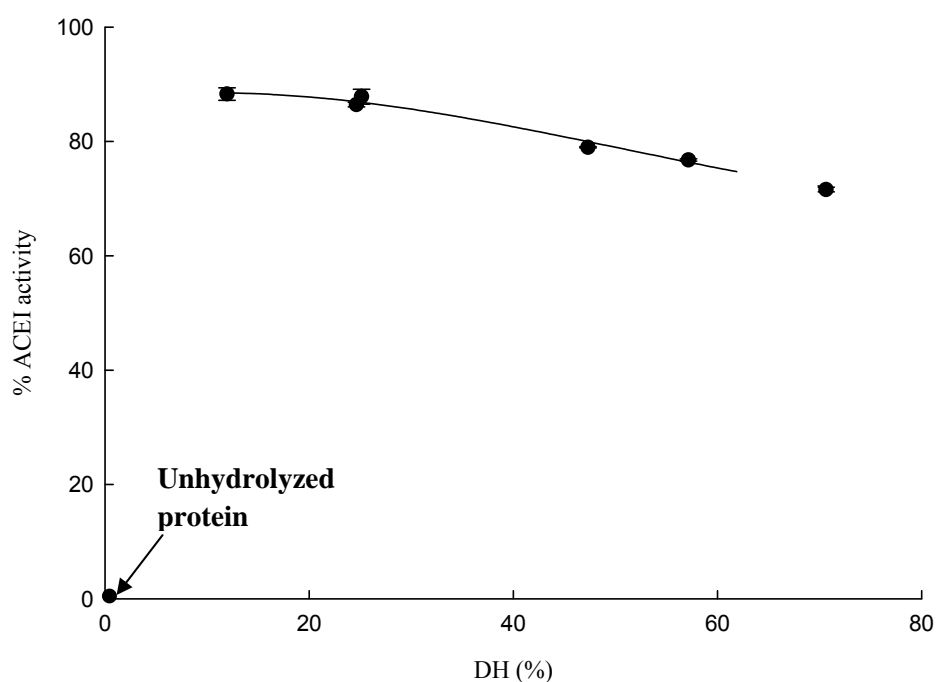


Figure 4.19 Angiotensin I-converting enzyme inhibitory activity of flaxseed protein hydrolysates in relation to degree of hydrolysis ($r^2=0.98$). A concentration of 0.67 mg hydrolysate/mL was used for the assays

Wu *et al.* (2006) reported that in long chain peptides (4-10 amino acid residues), the tetra peptide residue at the C-terminal end determines their structural requirement for effective inhibition of ACE activity. Therefore, it could be hypothesized that with the increase of DH, the exoproteases in Flavourzyme may be involved in removing amino acids from the C-terminal position of the ACEI peptides in FPHs making them less active resulting in a decrease in ACEI activity with further increase in DH.

The IC_{50} values for ACEI activity were calculated for three selected FPHs (DH: 11.94 %, 24.63 % and 25.13 %) possessing ACEI values above 85 % and are presented in Table 4.28. The ACEI values of these FPHs were significantly higher ($P<0.05$) than that

of all the other hydrolysates (Table 4.28). These FPHs had IC₅₀ values ranging between 0.07 and 0.09 mg/mL (Table 4.28) suggesting their ability to inhibit 50 % of ACE activity at a low concentration.

Table 4.28 The IC₅₀ for angiotensin I-converting enzyme inhibitory activity in flaxseed protein hydrolysates

E/S (LAPU/g protein)	Time of hydrolysis (h)	DH (%) ¹	ACEI activity (%) ¹	IC ₅₀ (mg of hydrolysate /mL) ¹
1.5	12.0	11.94 ± 4.1	88.29 ± 1.1	0.07 ± 0.0
47.5	0.7	24.63 ± 2.9	86.45 ± 0.4	0.09 ± 0.0
15.0	4.0	25.13 ± 1.6	87.83 ± 1.3	0.07 ± 0.0

¹Values are means ± standard deviations of duplicate analyses

E/S: Enzyme to substrate ratio; LAPU: Leucine amino peptidase units; DH: Degree of hydrolysis
ACEI: Angiotensin I-converting enzyme inhibitory

The IC₅₀ values for ACEI activity observed in the present study were greater than those reported for defatted flaxseed meal hydrolyzed with Alcalase and Thermolysin (Wu *et al.*, 2004). According to the process of Wu *et al.* (2004), Alcalase produced a hydrolysate with an IC₅₀ of 64.3 µg/mL, whereas the hydrolysate produced by Thermolysin had an IC₅₀ of 37.1 µg/mL for ACEI activity. The hydrolysate resulting from sequential hydrolysis of Thermolysin and Alcalase had an ACEI IC₅₀ of 34.2 µg/mL. However, these reported values were for hydrolyzed defatted flaxseed meal and not for the purified protein. When the meal is subjected to hydrolysis the resulting hydrolysate may contain various soluble, non-protein matrix components. For example, strong ACEI activities have been reported from plant derived flavanoids and proanthocyanidins (Hansen *et al.*, 1996; Wagner *et al.*, 1991) and guanosine derivatives (Kim *et al.*, 2003). Therefore, it is not certain whether the observed ACEI activity of the hydrolyzed flaxseed meal is solely due to peptides resulting from protein hydrolysis or any other component released from the complex chemical constituents of meal.

The ACEI IC_{50} values of FPHs reported in the present study were much lower compared to the crude hydrolysates of soy protein hydrolyzed with Alcalase (IC_{50} 0.34 mg/mL) (Wu & Ding, 2002), rapeseed protein hydrolyzed with Thermolysin (IC_{50} 0.32 mg/mL) (Marczak *et al.*, 2003), Alcalase-Flavourzyme hydrolyzed Kabuli and Desi chickpea proteins (0.316 and 0.228 mg/mL, respectively) and yellow pea protein (0.412 mg/mL) (Barbana & Boye, 2010), which indicated the high ACEI potential of FPHs generated in the present study. These differences in ACEI potency could be due to a various factors including differences in proteolytic enzymatic activity generating different peptide compositions in the hydrolysate, differences in parent protein structure, hydrolytic conditions (E/S, time, pH and temperature).

In a recent study, Nakahara *et al.* (2010) showed that a peptide enriched soy sauce like seasoning with an ACEI- IC_{50} value of 0.45 mg/mL demonstrated antihypertensive effects in SHR. Moreover, oral ingestion of a tryptic casein hydrolysate having an IC_{50} value of 0.166 mg/mL (Karaki *et al.*, 1990) and milk fermented with *Lactobacillus helveticus* (ACEI- IC_{50} : 0.16-0.26 mg/mL; Fuglsang *et al.*, 2002) showed significant blood pressure reduction in SHR. The IC_{50} value for the FPHs reported herein is therefore within the concentration range likely to mediate an antihypertensive effect.

The ACEI activity of the three FPHs with high activity (above 85 %) showed dose dependent relationships when plotted against the hydrolysate concentration. Figure 4.20 shows the relationship between ACEI activity and hydrolysate concentration for the hydrolysate having maximum ACE inhibition (IC_{50} : 0.07 mg/mL; DH: 11.94 %). There was an increase in the ACEI activity with the increasing hydrolysate concentration.

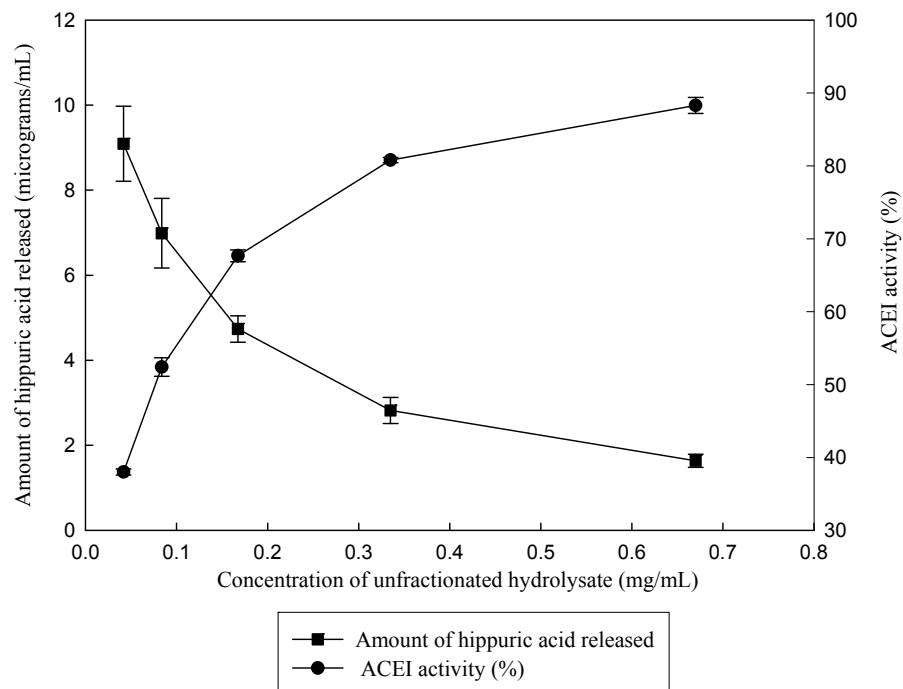


Figure 4.20 The Angiotensin I-converting enzyme inhibitory activity and amount of hippuric acid released in the presence of the flaxseed protein hydrolysate having 11.94 % degree of hydrolysis in the assay mixture

4.3.2.1.1 Angiotensin I-converting enzyme inhibitory pattern

The ACEI patterns of the three most potent ACEI- FPHs (DH: 11.94 %, 24.63 % and 25.13 %) were determined by the Lineweaver-Birk plots. The Lineweaver Birk plot for the hydrolysate with a DH of 11.94 % (ACEI IC_{50} : 0.07 mg/mL) is shown in Figure 4.21. The results indicated that these FPHs compete with Hip-His-Leu and prevents its binding with the active site of ACE causing ACE inhibition. Therefore, the FPHs were identified as competitive inhibitors of ACE with respect to Hip-His-Leu, which was used as the substrate for ACE in the assay.

Most food derived ACE inhibitors generated by microbial protease hydrolysis were found to be competitive inhibitors. Such inhibitors include alkaline protease hydrolysate of sardine muscle (Matsufuji *et al.*, 1994) and soy protein (Wu & Ding, 2002).

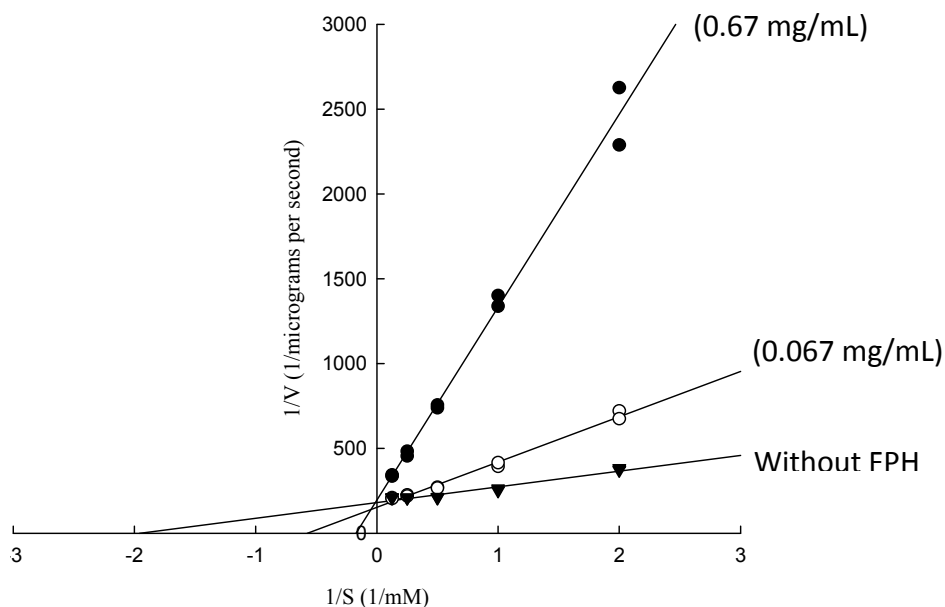


Figure 4.21 Lineweaver-Birk plots for angiotensin I-converting enzyme with or without flaxseed protein hydrolysate (Degree of hydrolysis: 11.9 %) having the highest angiotensin I-converting enzyme inhibitory activity. V: velocity of reaction measured as the amount of hippuric acid released per second, S: Substrate (Hip-His-Leu) concentration in the assay mixture

The non-competitive ACEI peptides also have been reported in some protein hydrolysates. The cationic peptide fraction of flaxseed protein hydrolysate generated by Alcalase hydrolysis and Thermolysin hydrolysate exhibited mixed type non-competitive ACE inhibition (Udenigwe *et al.*, 2009). Similar results have been reported by Udenigwe and Aluko (2010) for flaxseed protein hydrolysate with a high Fischer ratio generated by Thermolysin followed by pronase catalyzed hydrolysis. The authors highlighted that peptides in the hydrolysate inhibited ACE activity by binding both to the enzyme active site and allosteric site and the peptides interacted with ACE in both its free and substrate bound forms. The enzymes Thermolysin, Pronase and Alcalase have different specificities compared to Flavourzyme used in the present study. For example, Thermolysin cleaves proteins at the N terminal region of hydrophobic amino

acids (Phe, Tyr, Leu, Ile, Val). Alcalase is an endopeptidase and pronase possess both exopeptidase and endopeptidase activity (Udenigwe & Aluko, 2010). Different specificities of these enzymes with that of Flavourzyme could have generated different peptides in the flaxseed protein hydrolysates with different modes of ACE inhibition.

4.3.2.2 Antioxidant activities

The unfractionated FPHs were also screened for several *in vitro* antioxidant activities (scavenging of OH[•], O₂^{•-}, ability to inhibit linoleic acid oxidation and metal chelating activity) as given in Table 4.27.

4.3.2.2.1 Hydroxyl radical scavenging activity

The OH[•] scavenging activity of the FPHs (at 0.5 mg of hydrolysate /mL) ranged from 12.48 % to 22.08 % (Table 4.27). The maximum OH[•] scavenging activity (22.08 ± 0.3 %) was found in the FPH prepared with an E/S of 47.5 LAPU/g protein for 0.7 h (DH: 24.63 %). The OH[•] scavenging activity of this hydrolysate was significantly greater ($P < 0.05$) than that of hydrolysates prepared at 80 LAPU/ g protein for 20 h and 93.5 LAPU/ g protein for 12 h. It was not significantly different ($P < 0.05$) than the OH[•] scavenging activity of other FPHs. The hydrolysate prepared with an E/S of 80.0 LAPU/g of protein for 20 h (DH: 70.62 %) had the lowest OH[•] scavenging activity (12.48 ± 2.7 %) (Table 4.27). Similar to ACEI activity, there was no OH[•] scavenging activity in the unhydrolyzed flaxseed protein. Carnosine (0.5 mg/mL), a dipeptide with proven antioxidant activity, showed 63.5 % activity under the defined reaction conditions (Table 4.27).

Similar to ACEI activity, a plot of OH[•] scavenging activity of the FPHs against their DH values showed that increasing DH led to a decrease in the OH[•] scavenging ability (Figure 4.22). The OH[•] scavenging activity was greater in FPHs possessing lower DH and higher PCL compared to those with higher DH and lower PCL (Figure 4.22, Table 4.27). This suggests that the relatively long chain peptides acted as potent OH[•] scavengers.

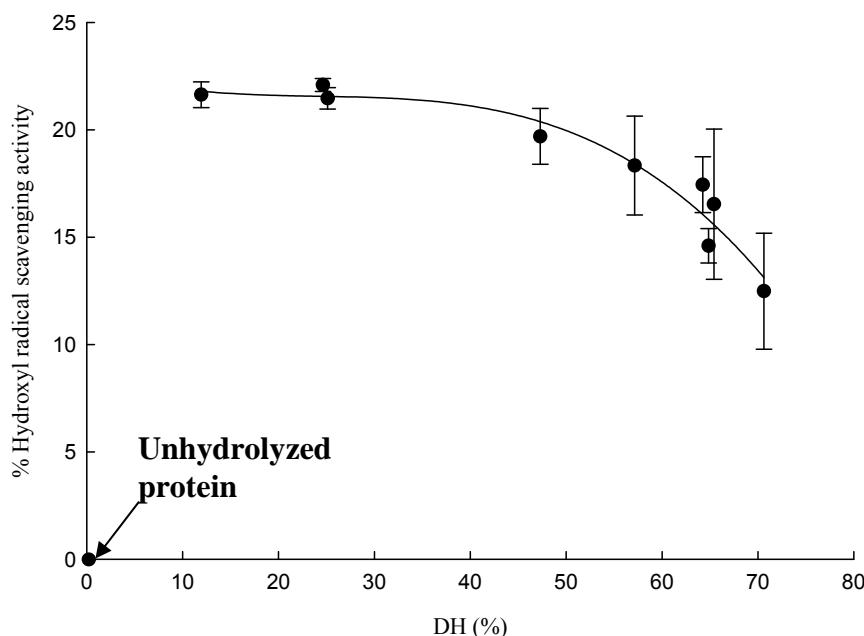


Figure 4.22 Hydroxyl radical scavenging activity of flaxseed protein hydrolysates in relation to degree of hydrolysis ($r^2=0.94$). Concentration of 0.5 mg hydrolysate/mL was used for the assays.

The long chain peptides in hydrolysates of lower DH may have been further hydrolyzed into short chain peptides thereby reducing the OH^\bullet scavenging activity. Previous studies on fish protein hydrolysates also have shown a decrease in OH^\bullet scavenging activity above a certain DH (You *et al.*, 2009). Long chain antioxidant peptides (5-16 amino acid residues) also have been isolated from hydrolyzed soy protein (Chen *et al.*, 1995). In contrast to the findings of the current study, Flavourzyme hydrolyzed soy concentrates had an increase in OH^\bullet scavenging activity with increased DH (Moure *et al.*, 2006). The FPH that showed the highest OH^\bullet scavenging activity (22.08 %) was selected for further studies.

Figure 4.23 shows the variation of OH^\bullet scavenging activity of the FPH possessing maximum activity (22.08 %; DH: 24.63 %) in relation to the hydrolysate concentration. Similar to ACEI activity, there was a dose dependent relationship between the OH^\bullet scavenging activity and FPH concentration.

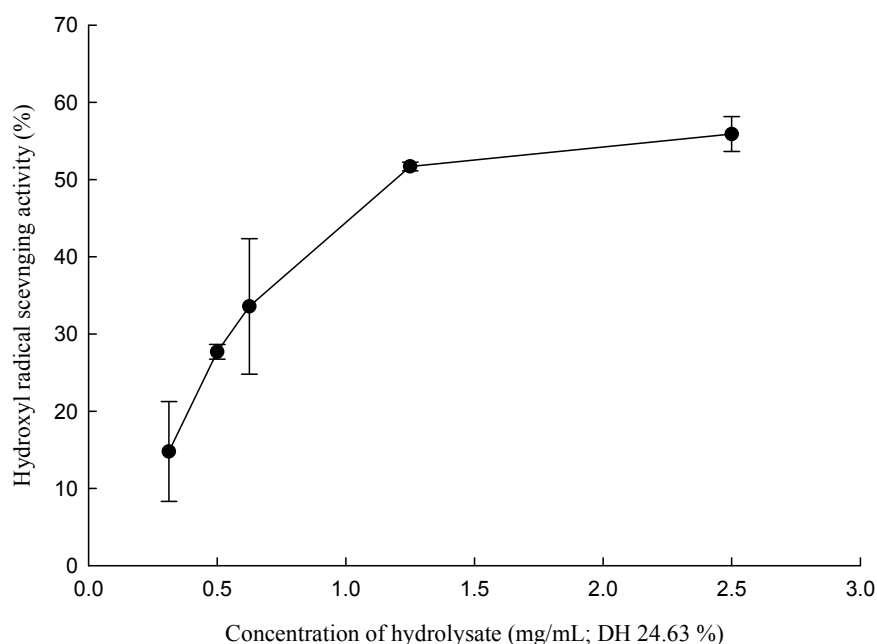


Figure 4.23 Hydroxyl radical scavenging activity of the flaxseed protein hydrolysate having 24.63 % degree of hydrolysis in relation to hydrolysate concentration

The increase in activity per unit change in FPH concentration was greater at concentrations below 1.25 mg/mL and was lower at concentrations above that limit (Figure 4.23).

The FPHs, which had above 85 % ACEI activity (DH: 11.94 %, 24.63 % and 25.13 %) were the ones showing the highest OH[•] scavenging activities (above 20 %). Therefore, the IC₅₀ values of those FPHs were calculated for OH[•] scavenging activity and they ranged from 2.03 ± 0.5 to 3.06 ± 2.4 mg/mL (Table 4.29). The IC₅₀ values shown by the FPHs were greater than that for ACEI activity (Table 4.28). This further indicated that the FPHs had a lower potential for OH[•] scavenging compared to ACE inhibition.

Previous studies support the presence as well as absence of OH[•] scavenging activity of protein hydrolysates obtained by microbial protease catalyzed hydrolysis. Flavourzyme hydrolyzed soy concentrate upon passing through a 50 kDa membrane previously

showed an activity of 21.81 % (concentration used: 0.1 mg protein/mL) at a DH of 28.8 % and this value was found to be similar to the OH[•] scavenging activity shown by FPH at similar DH (Moure *et al.*, 2006). In a recent study, Pownall *et al.* (2010) reported the absence of OH[•] scavenging activity in pea protein hydrolyzed with Thermolysin.

Table 4.29 The IC₅₀ for hydroxyl radical scavenging activity of flaxseed protein hydrolysates

E/S (LAPU/g protein)	Time of hydrolysis (h)	DH (%) ¹	OH [•] scavenging activity (%) ¹	IC ₅₀ (mg hydrolysate/mL) ¹
1.5	12.0	11.94 ± 4.2	21.63 ± 0.6	3.06 ± 2.4
47.5	0.7	24.63 ± 2.9	22.08 ± 0.3	1.56 ± 0.8
15.0	4.0	25.13 ± 1.6	21.46 ± 0.5	2.03 ± 0.5

¹ Values are means ± standard deviations of duplicate analyses

E/S: Enzyme to substrate ratio; LAPU: Leucine aminopeptidase units

DH: Degree of hydrolysis; OH[•] Hydroxyl radical

Rapeseed peptides generated by Alcalase hydrolysis had a weaker

Rapeseed peptides generated by Alcalase hydrolysis had a weaker OH[•] scavenging activity (IC₅₀ of 2.53 to 6.79 mg/mL) (Zhang *et al.*, 2008) compared to the FPHs generated in current study. Egg yolk protein hydrolysate prepared by sequential hydrolysis by Orientase and Protease from *Bacillus* spp., had a greater OH[•] scavenging activity (91.7 %) than the maximum activity reported by us at 0.5 % concentration (Sakanaka & Tachibana, 2006). Udenigwe and group (Udenigwe *et al.*, 2009; Udenigwe & Aluko, 2010) reported OH[•] scavenging activity of flaxseed protein hydrolysates. In one study (Udenigwe *et al.*, 2009), LMW (< 1kDa) cationic peptide fractions obtained from Alcalase, ficin, pancreatin, papain, Thermolysin and trypsin, were evaluated for OH[•] scavenging ability. The least cationic peptide fraction of Alcalase hydrolysis (IC₅₀: 0.08 mg protein/mL), ficin (0.26 mg protein/mL), trypsin (IC₅₀: 0.01 mg protein/mL), papain (IC₅₀: 0.51 mg protein/mL), Thermolysin (IC₅₀: 0.05 mg protein/mL) and pancreatin (IC₅₀: 0.07 mg protein/mL) exhibited high OH[•] scavenging activity. Sequential hydrolysis of flaxseed protein by Thermolysin followed by pronase, resulted

in maximum scavenging of 31.5 % (5 mg protein/mL), which is a moderate OH[•] scavenging activity (Udenigwe & Aluko, 2010). Very low levels of Phe residues in the peptide mixture, was considered a contributing factor for the moderate OH[•] scavenging activity in flaxseed protein hydrolysates (Udenigwe & Aluko, 2010). Formation of a stable *o*-, *m*- or *p*-Tyr by trapping a OH[•] and hydroxylation of aromatic ring have been reported as the reason for strong OH[•] scavenging ability of Phe (Sun *et al.*, 1993). Figure 4.23 showed a OH[•] scavenging activity of 31.5 % by the Flavourzyme catalyzed FPH at <1 mg/mL hydrolysate concentration.

In previous studies, OH[•] scavenging activity of hydrolyzed flaxseed protein have been determined upon further modification of the hydrolysates. These modifications include removal of HMW peptides and enrichment of the hydrolysates with cationic peptides (Udenigwe *et al.*, 2009), removal of aromatic amino acid containing peptides and recovering BCAA containing peptides by passing the hydrolysate through activated carbon column (Udenigwe & Aluko, 2010). In contrast, the hydrolysate that we used was crude in nature without concentrating specific peptides.

4.3.2.2.2 Superoxide radical scavenging activity

The O₂^{•-} scavenging activity of the FPHs (0.67 mg of hydrolysate/mL) ranged from 26.33 ± 2.5 to 39.41 ± 1.9 % (Table 4.27). The highest O₂^{•-} scavenging activity was observed when the protein was hydrolyzed at E/S of 93.5 LAPU/g for 12 h (DH: 64.86 %). This was significantly higher (*P*<0.05) than the activities shown by the FPHs prepared by treating flaxseed protein with 15 LAPU/g for 4 h, 47.5 LAPU/g for 0.7 h and with 80 LAPU/g for 4 h and 20 h (Table 4.27). The unhydrolyzed protein did not show any O₂^{•-} scavenging activity. Therefore, it could be hypothesized that O₂^{•-} scavenging peptides are released during hydrolysis of flaxseed protein by Flavourzyme, which increased the O₂^{•-} scavenging activity of FPHs. However, all hydrolysates had lower O₂^{•-} scavenging activity than ascorbic acid (62.24 ± 1.1 %), which was used as the standard (Table 4.27).

According to published research, both the size and structure of the peptides are determinants of antioxidant activity. Chen *et al.* (1995) found that the presence of His, Pro and Tyr in the sequence as well as the hydrophobic amino acids, Val or Leu at the N terminal are the main structural features of antioxidant peptides obtained from soy bean protein digests. However, unlike the OH^\bullet scavenging activity, the $\text{O}_2^{\bullet-}$ scavenging activity of FPHs did not show any correlation with the DH (Figure 4.24).

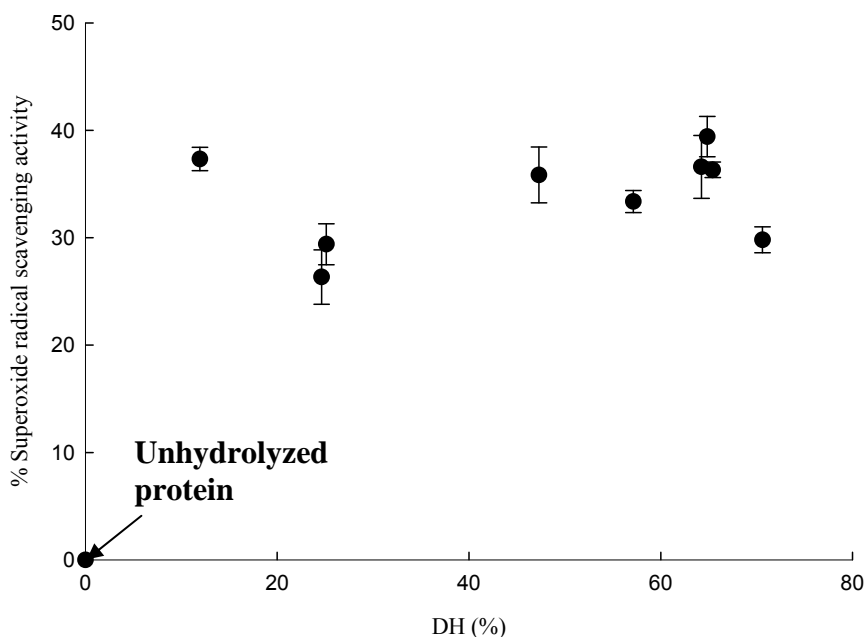


Figure 4.24 Superoxide radical scavenging activity of flaxseed protein hydrolysates in relation to degree of hydrolysis. A concentration of 0.67 mg hydrolysate/mL was used for the assays

It is possible that the structure of peptides play a role in determining the $\text{O}_2^{\bullet-}$ scavenging activity of the FPHs and not the PCL. The hydrolysate that showed the highest $\text{O}_2^{\bullet-}$ scavenging activity (39.41 %) was selected for further studies.

The FPH with the highest $\text{O}_2^{\bullet-}$ scavenging activity (39.41 %; DH of 64.86 %) was further investigated to determine the IC_{50} for $\text{O}_2^{\bullet-}$ scavenging activity. The FPH had an IC_{50} of 1.2 mg of hydrolysate /mL. It showed an increasing $\text{O}_2^{\bullet-}$ scavenging activity with the increase in concentration (Figure 4.25).

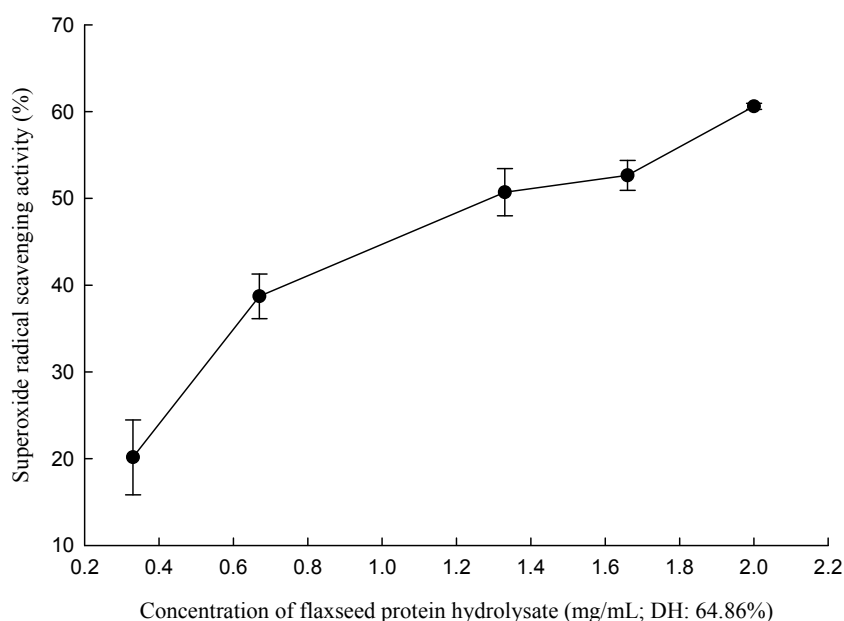


Figure 4.25 Superoxide radical scavenging activity of the flaxseed protein hydrolysate having 64.86 % degree of hydrolysis in relation to hydrolysate concentration

There are many biological reactions generating $O_2^{\bullet-}$, which is highly toxic. Superoxide radical itself is a poorly reactive radical. However, it causes indirect cell damages by generating highly reactive oxygen species such as singlet oxygen and OH^{\bullet} (Benzie, 2000). Therefore, study of the scavenging ability of $O_2^{\bullet-}$ is important. The flaxseed protein hydrolysate with high Fischer ratio prepared by Thermolysin-pronase hydrolysis (Udenigwe & Aluko 2010) also exhibited $O_2^{\bullet-}$ scavenging activity with a reported IC_{50} value of 1.67 ± 0.19 mg protein/mL.

The unfractionated FPH with highest $O_2^{\bullet-}$ scavenging activity resulted in an IC_{50} of 1.2 mg of hydrolysate /mL and hence had a greater activity than the hydrolysate prepared by Udenigwe and Aluko (2010). Also the highest $O_2^{\bullet-}$ scavenging activity shown by the FPH was greater than the cationic peptide rich fraction of flaxseed protein hydrolysates generated by hydrolyzing flaxseed protein with seven different proteases as described by Udenigwe *et al.* (2009). As reported by these authors, the flaxseed protein hydrolysates prepared by pepsin, trypsin, Thermolysin and pancreatin as well as the least cationic

fraction generated by Alcalase hydrolysis had no $O_2^{\bullet-}$ scavenging activity. The IC_{50} for $O_2^{\bullet-}$ scavenging activity for hydrolysates prepared from Alcalase, ficin and papain ranged from 1.66 to 3.98 mg protein/mL, which was greater than that observed in current study (1.2 mg of hydrolysate/mL). Our results indicate that it is possible to generate FPHs with higher $O_2^{\bullet-}$ scavenging ability using Flavourzyme compared to the above mentioned proteolytic enzymes.

The $O_2^{\bullet-}$ scavenging activity of FPHs was weaker than that of hydrolysates of wheat germ prepared with Alcalase (IC_{50} : 0.4 mg/mL) (Zhu *et al.*, 2006), egg yolk protein prepared with proteinase from *Bacillus* sp. (90 % $O_2^{\bullet-}$ scavenging activity at 0.5 % concentration; Sakanaka & Tachibana, 2006) and Alcalase hydrolysates of alfalfa leaf protein (67 % at 0.9 mg/mL) (Xie *et al.*, 2008) and rapeseed protein (IC_{50} : 1.05 mg/mL) (Pan *et al.*, 2009).

4.3.2.2.3 Ability to inhibit linoleic acid oxidation

All the FPHs showed the ability to inhibit linoleic acid oxidation (Table 4.27). In some FPHs it was greater than that of the standard (ascorbic acid) used (63.87 ± 0.5 %). The highest activity (94.33 ± 4.5 %) was found in the FPH prepared under the conditions; 1.5 LAPU/ g protein for 12 h. This FPH had the lowest DH (11.94 %; PCL: 9.1). There was no significant difference between this FPH and that prepared using 93.5 LAPU/g protein for 12 h for inhibition of linoleic acid oxidation (88.17 ± 1.4 %). The lowest activity (0.71 ± 4.7 %) was in the FPH with the highest DH (70.6 %; PCL: 1.4) (Table 4.27). Unlike other bioactivities tested, the unhydrolyzed flaxseed protein also inhibited linoleic acid oxidation (19.62 ± 1.6 %; Table 4.27). Hydrolysis by Flavourzyme increased the inhibition of linoleic acid oxidation in all the FPHs except the FPH prepared using E/S of 80 LAPU/ g protein for 20 h (Table 4.27). The results further indicated that the FPHs with long chain peptides (PCL~9) were potent inhibitors of linoleic acid oxidation (Table 4.27). However, when plotted in a graph (Figure 4.26) the activity of FPHs did not show any correlation with the DH similar to the $O_2^{\bullet-}$ scavenging activity (Figure 4.25).

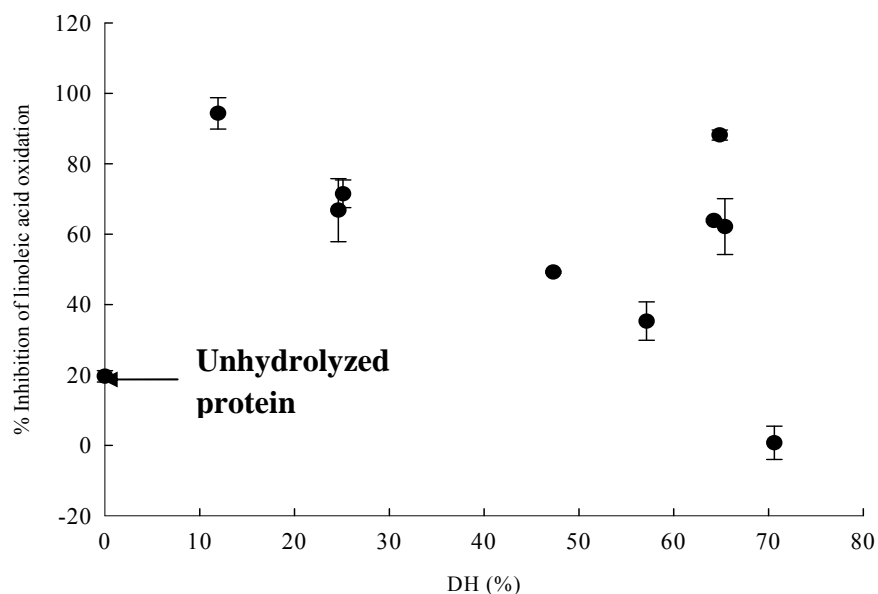


Figure 4.26 Inhibition of linoleic acid oxidation of flaxseed protein hydrolysates in relation to degree of hydrolysis. Concentration of 0.67 mg hydrolysate/mL was used for the assays

As stated previously, the lack of a direct relationship between antioxidant activity and DH suggested that the specific composition (e.g., type of peptides, ratio of different free amino acids) was an important factor in determining the ability of FPHs to inhibit linoleic acid oxidation. The hydrophobic amino acids such as Phe and Gly in peptides were reported to contribute to inhibition of lipid peroxidation as they make the peptides soluble in lipid and thereby facilitate better interaction with radical species. Moreover, the activity of His containing peptides has also been reported to act against lipid peroxidation (Chen *et al.*, 1998). This activity may be due to presence of an imidazole ring in His structure, which may be involved in hydrogen donation and lipid radical trapping ability (Chen *et al.*, 1998).

The FPH that possessed the maximum ability to inhibit linoleic acid oxidation (94.33 %, DH: 11.94 %) was selected for further studies. The IC_{50} of this FPH was 0.4 mg/mL. As shown in Figure 4.27, there was a dose dependent relationship between the hydrolysate

and the percent inhibition of linoleic acid oxidation. However, at concentrations below $\sim <0.3$ mg/mL, the FPH did not show any activity. In the present study, the ferric thiocyanate assay was first used with a control sample (with no hydrolysate) to determine the time taken to form maximum level of peroxides of linoleic acid oxidation. In this assay the increase in absorbance at 507 nm was used as an indication of the increase in concentration of peroxides formed.

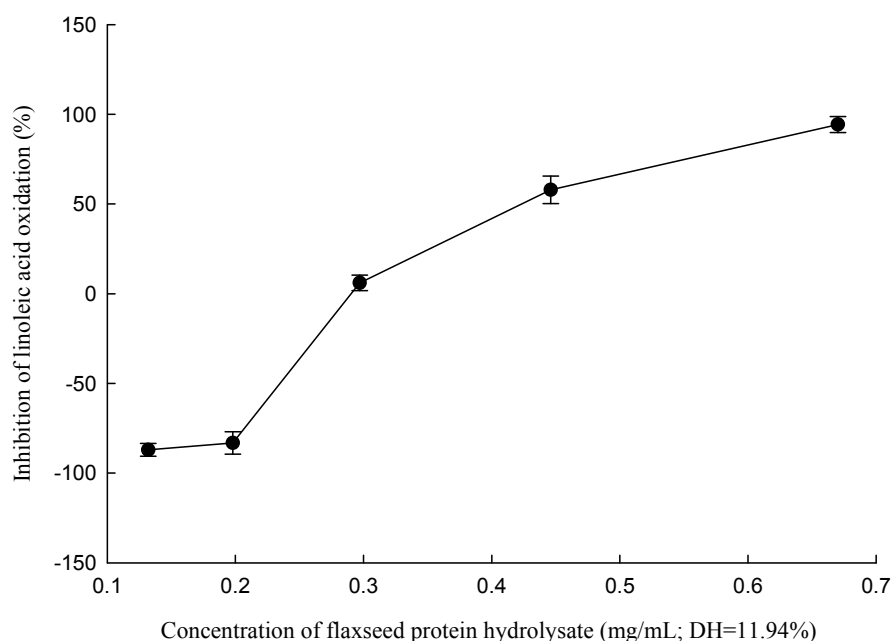


Figure 4.27 The inhibition of linoleic acid oxidation of the flaxseed protein hydrolysate having 11.94 % degree of hydrolysis in relation to hydrolysate concentration

The maximum peroxide formation was observed at 48 h (results not shown) and decreased thereafter. As peroxides are unstable, they gradually decompose into secondary oxidation products (Chen *et al.*, 1996). This led to a decrease in absorbance (at 507 nm) after the second day of incubation in the current study. As oxidation proceeds, peroxides are gradually decomposed into LMW compounds that can be measured using the TBA reagent (Chen *et al.*, 2007). As shown by Chen *et al.*, (2007), peanut protein hydrolyzed with Alcalase inhibits oxidation of linoleic acid by 79 % at 20

mg/mL. This concentration was much higher than that used in the present study (0.67 mg/mL). Therefore, the Flavourzyme hydrolyzed flaxseed protein (especially that having DH of 11.94 %) had a greater ability to inhibit linoleic acid oxidation compared to the peanut protein hydrolysate reported by Chen *et al.* (2007). A marked inhibition of linoleic acid oxidation also has been shown by a gelatin hydrolysate of Alaska pollack skin prepared with Alcalase followed by Pronase E (58 % inhibition; Kim *et al.*, 2001). According to Dong *et al.* (2008), Alcalase catalyzed silver carp protein hydrolysate showed higher inhibition of linoleic acid oxidation than when Flavourzyme was used. The ability to inhibit linoleic acid oxidation by unhydrolyzed flaxseed protein and FPH has not been previously reported.

4.3.2.2.4 Metal chelating activity

In the present study, the ability of FPHs to chelate ferrous ions was investigated. Similar to inhibition of linoleic acid oxidation, the unhydrolyzed flaxseed protein had 29.57 ± 0.7 % metal chelating activity. Interestingly, the metal chelating activities of all the FPHs were lower than that of the unhydrolyzed protein (Table 4.27). Therefore, the hydrolysis of flaxseed protein by Flavourzyme reduced its metal chelating activity. This indicated that hydrolysis of flaxseed protein by Flavourzyme may not release metal chelating peptides or that the activity was lost due to hydrolysis.

Transitional metal ions such as Fe^{2+} act as prooxidants, and can catalyze the generation of reactive oxygen species such as OH^{\bullet} which, in turn, oxidize unsaturated lipids (Burkitt, 2001; Stohs & Bagchi, 1995). Therefore, chelation of metal ions will prevent oxidative damage leading to cardioprotection. Metal chelating activity was previously shown by various protein hydrolysates including Alcalase hydrolyzed wheat germ protein (Zhu *et al.*, 2006), Thermolysin hydrolyzed pea protein (Pownall *et al.*, 2010), porcine collagen hydrolyzed by a mixture of bovine pancreas, protease of *Streptomyces* and protease of *Bacillus* spp (Li *et al.*, 2007), porcine hemoglobin (Chang *et al.*, 2007) and silver carp protein (Dong *et al.*, 2008) prepared with Alcalase and Flavourzyme. The optimal metal chelating activity involves aliphatic compounds, where a five-member ring is formed, composed of the metal ion and two chelating ligands (Miller *et*

al., 1990). Hydrophobic amino acids as well as His were rich in hydrolysates with metal chelating activity (Megias *et al.*, 2008; Pownall *et al.*, 2010). It was suggested that the imidazole ring is responsible for the strong metal chelating activity of the amino acid, His (Megias *et al.*, 2008). In addition, the importance of N-terminal peptide sequence of peptides (Chen *et al.*, 1998), presence of amino acids such as Tyr, Met and Trp (Amarowicz, 2008) as well as acidic and/or basic amino acids (Suetsuna *et al.*, 2000), in the metal chelating activity has been reported.

According to the results, it is possible that the amino acid compositions of peptides in FPHs prepared in the current study were not favoring their ability to chelate metals. The flaxseed protein used for this study had a relatively high amount of acidic amino acids such as Asp and Glu as well as the basic amino acid Arg (See Table 4.6 shown in the results and discussion chapter of this thesis), which could be the reason for its high metal chelating activity. It could be hypothesized that Flavourzyme had hydrolyzed the flaxseed protein leaving acidic amino acid containing peptide chains intact in the unhydrolyzed residue, resulting in FPHs with low metal chelating activity.

The chelation of metal ions can decrease the amount of free iron available to participate in the Fenton reaction used for determination of OH[•] scavenging activity and ultimately decrease the formation of the OH[•] (Halliwell & Gutteridge, 1990). Therefore, if a hydrolysate shows both OH[•] scavenging activity and metal chelating activity it is important to clarify that the OH[•] scavenging activity observed was not due to chelation of iron. The absence of metal chelating activity of FPHs further confirmed that the results obtained in deoxy ribose assay was due to their ability to scavenge OH[•] generated by the Fenton reaction.

The metal chelating activity of the hydrolysates was not studied further and the IC₅₀ was not determined.

4.3.2.2.5 Antithrombotic activity

The antithrombotic activity of FPHs, unhydrolyzed protein and the standard antithrombin, are shown in Table 4.27. Most hydrolysates showed minimal or no antithrombotic activity. The unhydrolyzed proteins and blanks also did not exhibit antithrombotic activity indicating that the activity shown was due to products generated by Flavourzyme catalyzed hydrolysis of flaxseed protein. The FPHs possessing antithrombotic activity showed comparatively lower activity at the concentration used, which ranged from 8.39 ± 1.9 (E/S: 1.5 LAPU/ g protein; Time: 12 h; DH: 11.94 %) to 19.83 ± 2.1 % (E/S: 47.5LAPU/ g protein; Time: 0.7 h; DH: 24.6 %). The hydrolysates below and above 24.63 % DH had low or no antithrombotic activity.

In the current study, the inhibition of thrombin catalyzed coagulation of fibrinogen was measured and expressed as antithrombotic activity (Table 4.27). Antithrombotic peptides, derived from food proteins, which show nearly no toxic effect, have broad application in cardiovascular disease prevention and treatment. Antithrombotic activity was previously observed in a few studies on hydrolyzed food proteins. They included fermented soy foods such as pronase digest of mature natto (IC_{50} : 36.2 to 162.1 μ M) and kidney membrane protease digests of matured tempeh (IC_{50} : 9.8 to 148.2 μ M) (Gibbs *et al.*, 2004). An antithrombotic peptide in the kidney membrane protease digest of tempeh had Arg–Pro sequence and had 63 % homology to a previously reported thrombin inhibitor, hirutinin (DiMiao *et al.*, 1992). Rapeseed peptides derived from Alcalase hydrolysis showed marked inhibitory activities on the thrombin-catalyzed coagulation of fibrinogen at certain concentrations (e.g. 90 % inhibition at 30 and 40 mg/mL) although their inhibitory effects were not dose-dependent. The antithrombotic activity of rapeseed peptides were lower than heparin (standard used) and was higher than that of egg white hydrolysate (Yang *et al.*, 2007), when used at the same concentration.

Due to the weaker antithrombotic activity shown by the hydrolysates compared to that of the standard, antithrombin (100 % at the used concentration of 0.67 mg/mL), further analysis of this bioactivity was not carried out.

4.3.2.2.6 Bile acid binding ability

The bile acid binding ability of FPHs and unhydrolyzed protein was determined *in vitro* using five individual bile acids (SCDC, SC, STC, SGC and SDC). In contrast to other bioactivities assayed, all the hydrolyzed flaxseed proteins (0.67 mg/mL) demonstrated no or very low bile acid binding ability (results not shown). Therefore, the IC₅₀ value was not determined for bile acid binding. Interestingly, the unhydrolyzed flaxseed protein (0.67 mg/mL) bound all five bile acids; 15.39 ± 4.8 %, 17.30 ± 0.6 %, 59.76 ± 2.8 %, 37.95 ± 1.31 % and 32.20 ± 3.1 % of SCDC, SC, STC, SGC and SDC, respectively (Figure 4.28). The sample blanks (flaxseed protein isolate maintained at 50 °C without adding Flavourzyme, no hydrolysis occurred, DH: 0 %) possessed bile acid binding abilities, which were similar or greater than that of the hydrolyzed protein (results not shown). Therefore, the bile acid binding ability observed in the FPHs, even in very minute amounts, could not be attributed to Flavourzyme catalyzed hydrolysis.

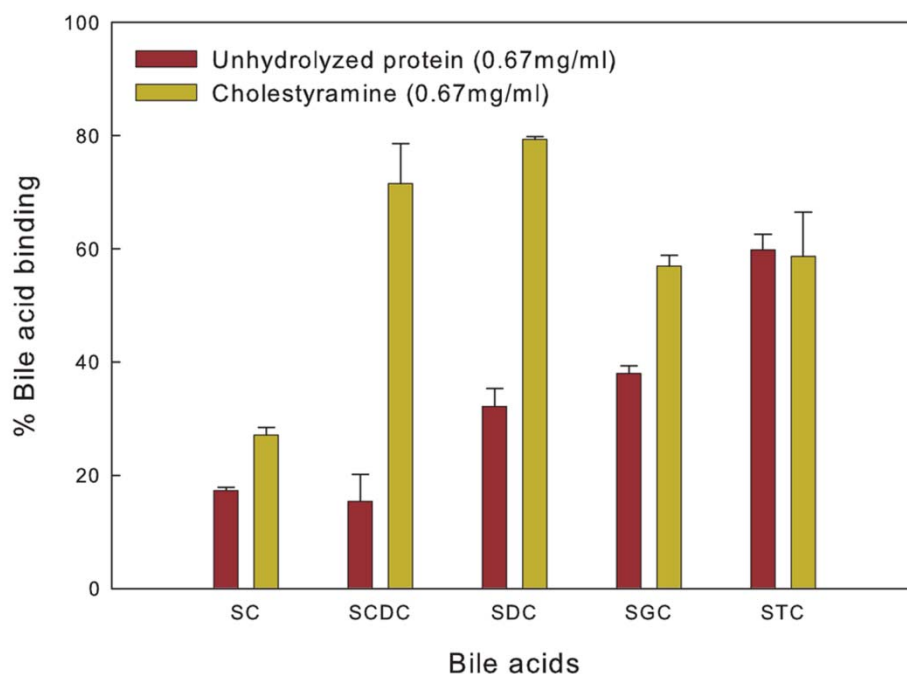


Figure 4.28 Bile acid binding ability of unhydrolyzed flaxseed protein and cholestyramine (SC=Sodium Cholate, SCDC=Sodium Chenodeoxycholate, SDC=Sodium Deoxycholate, SGC=Sodium Glycocholate, STC=Sodium Taurocholate; Flaxseed protein hydrolysates showed no binding effect)

The ability of cholestyramine, the bile acid binding and cholesterol-lowering drug, to bind the individual bile acids was also tested. Cholestyramine (0.67 mg/mL) bound 71.44 ± 7.1 % SCDC, 27.07 ± 1.4 % SC, 58.59 ± 7.8 % STC, 56.94 ± 1.87 % SGC and 79.33 ± 0.43 % of SDC. The ability to bind STC was greater, but not significantly different in unhydrolyzed flaxseed protein than cholestyramine (Figure 4.28). In this study, the majority of FPHs possessed short PCL due to extensive hydrolysis of flaxseed protein by Flavourzyme. The inability of peptides with short PCL to bind bile acids may be the reason for reduction of the bile acid binding ability upon hydrolysis. It has been demonstrated that the bile acid binding capacity of a peptide was closely related to its hydrophobic nature. Such peptides tend to bind with bile acids by hydrophobic interactions and get excreted into feces with bile acids (Higaki *et al.*, 2006). Therefore, the bile acid binding ability of unhydrolyzed flaxseed protein also could be due to its hydrophobic nature.

The values for bile acid binding ability of cholestyramine observed in the present study deviated from the previously reported values (Yoshi-Starke & Wasche, 2004) and this could be attributed to the concentration of cholestyramine and the differences in the assay kit used. Only a few studies have reported bile acid binding of proteins and their hydrolysates *in vitro*, including soy protein and wheat gluten (Kahlon & Woodruff, 2002), lupin protein and hydrolysates generated by pepsin as well as pepsin-pancreatin hydrolysis (Yoshi-Starke & Wasche, 2004), rapeseed protein (Yoshie-Stark *et al.* 2006) buckwheat protein and its pepsin-pancreatin digests (Ma & Xiong, 2009).

Higaki *et al.* (2006) showed that the HMW fraction of soy protein digested with pepsin followed by pancreatin was capable of binding bile acids *in vivo*. In a previous study, lupin protein isolate (1 mg/mL, protein content 91.44 %) was able to bind 55.8 % SCDC, 54.4 % SC, 40.9 % STC, 63.9 % SGC and 58.4 % SDC. The bile acid binding ability of flaxseed protein was lower than that of lupin protein isolate except for STC binding (Yoshi-Starke & Wasche, 2004). Similar to present findings, Yoshi-Starke and Wasche (2004) also reported greater bile acid binding of lupin protein compared to cholestyramine. Yoshie-Stark *et al.* (2008) reported that rapeseed protein isolate (1

mg/mL, protein content 70.8 %) had a 5.77 % SC binding and 10 % SDC binding activity, which was much lower than the values shown by flaxseed protein in the present study.

However, the simulated GI digestion carried out in the study I of this thesis using dynamic model indicated that flaxseed protein when hydrolyzed sequentially by GI proteases (pepsin and pancreatin), generates a digest with a DH of 46.78 % (See section 4.5.6.2 under the results and discussion chapter of this thesis). Hence, it is possible that the ability of intact flaxseed protein to bind bile acids is modified upon GI digestion. This would be the same for other proteins such as wheat gluten (Kahlon & Woodruff, 2002) and rapeseed protein (Yoshie-Stark *et al.*, 2006) whose bile acid binding ability in the digested form has not been reported.

Since none of the FPHs showed considerable bile acid binding activity except the unhydrolyzed protein, this bioactivity was not studied further.

4.3.3 Identification of precursor proteins of bioactive peptides based on degradation pattern of proteins

Figure 4.29 shows the SDS-PAGE patterns (NR) of the protein isolate residues that remained upon Flavourzyme catalyzed hydrolysis. Of the major protein bands observed in the isolated flaxseed protein, the band with a molecular mass of around 48 kDa was highly susceptible for Flavourzyme catalyzed hydrolysis. At the DH of 11.94 %, this polypeptide band lost 90 % of its pixel intensity and was completely degraded when the DH was above 11.94 % (Table 4.30). The polypeptide band of ~41 kDa was visible in all the residues indicating its resistance to Flavourzyme hydrolysis. This was in contrast to protein band degradation observed in study I where simulated GI digestion caused complete degradation of the two HMW protein bands (48 and 41 kDa). At all the DH values the ~41 kDa band showed greater pixel intensity than that of the unhydrolyzed residue (Table 4.30). Therefore, in contrast to unhydrolyzed protein, the ~ 41 kDa band of samples at different DH may contain the degraded products of other polypeptides especially those of 48 kDa.

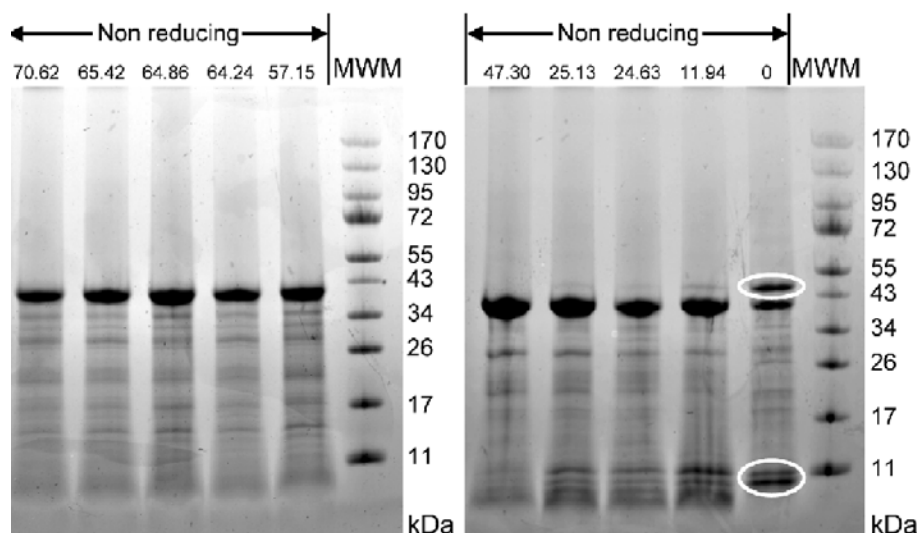


Figure 4.29 Electrophoretic separation of flaxseed protein isolate and residues remaining after hydrolysis under non reducing conditions. Lanes are labelled with degree of hydrolysis %; 0 degree of hydrolysis indicates unhydrolyzed flaxseed protein isolated at pH 3.8; MWM: molecular weight markers. Gels used were gradient mini gels (resolving 8-25% T and 2% C, stacking zone 4.5 %T and 3 %C, 43×50×0.45 mm, polyacrylamide gels cast on GelBond® plastic backing, buffer 0.112 M acetate, 0.112 M Tris, pH 6.4, Tris-Tricine running buffer). Each well was loaded with 5 µg protein

The electrophoregrams of the unhydrolyzed residue at different DH are shown in Figures 4.30 and 4.31. Above the DH of 11.94 %, there were new polypeptide bands that appeared with apparent mass below 10 kDa, most likely the hydrolytic products of the parent protein of the isolate (Figures 4.30 and 4.31). The polypeptide bands of 13 and 12 kDa were completely degraded above 25.13 % DH leaving the insoluble LMW hydrolytic products (Figures 4.29, 4.30 and Table 4.30).

Results indicated that flaxseed proteins of 43 kDa and lower molecular masses were less susceptible to Flavourzyme catalyzed hydrolysis when compared to the protein band of 41 kDa. The indigestible residues corresponding to DH between 57.15 % and

70.6 2 % (Figures 4.29, and 4.30) showed identical electrophoretic patterns with ~ 41 kDa protein band as the major remaining protein.

Table 4.30 Difference (%) in polypeptide band intensity of the remaining flaxseed protein residues at the end of Flavourzyme catalyzed hydrolysis compared to the unhydrolyzed protein.

DH (%)	Percentage difference in pixel intensity ¹ of polypeptide bands					
	48 kDa	41 kDa	29 kDa	20 kDa	13 kDa	12 kDa
11.94	-90.6	+160.2	-43.81	-73.2	-3.5	-64.5
24.63	-100	+125.3	-13.0	-38.9	-44.1	-84.4
25.13	-100	+194.8	+1.9	-39.8	-39.2	-77.9
47.30	-100	+232.7	+44.27	-100	-100	-100
57.15	-100	+142.2	+105.7	+103.2	-100	-100
64.24	-100	+161.7	+39.8	-40.9	-100	-100
64.86	-100	+120.9	+52.4	+31.0	-100	-100
65.42	-100	+82.7	+17.8	-21.5	-100	-100
70.62	-100	+127.5	+10.1	-15.8	-100	-100

DH: Degree of hydrolysis

¹Negative and positive values indicate respectively a reduction and gain of pixel intensity of polypeptide bands compared to the initiation of digestion (e.g. -100 indicates a complete loss of a polypeptide band). Band molecular masses refer to the Figure 4.30.

The degradation of protein bands at different DH levels were compared with the bioactivities shown by the hydrolysates at those DH levels. As shown by SDS-PAGE (Figures 4.29 and 4.30), a significant reduction in the intensity of protein band of ~ 48 kDa occurred when the DH achieved 11.94 %. The highest ACEI activity and highest inhibition of linoleic acid oxidation were shown by the hydrolysate with DH of 11.94 % (Table 4.27). Therefore, this suggests that peptides generated by hydrolysis of 48 kDa protein band may have contributed to ACEI activity and inhibition of linoleic acid oxidation shown by the hydrolysates. The protein band of 48 kDa could be acting as the precursor protein for generation of bioactive peptides with ACEI activity and inhibition of linoleic acid oxidation.

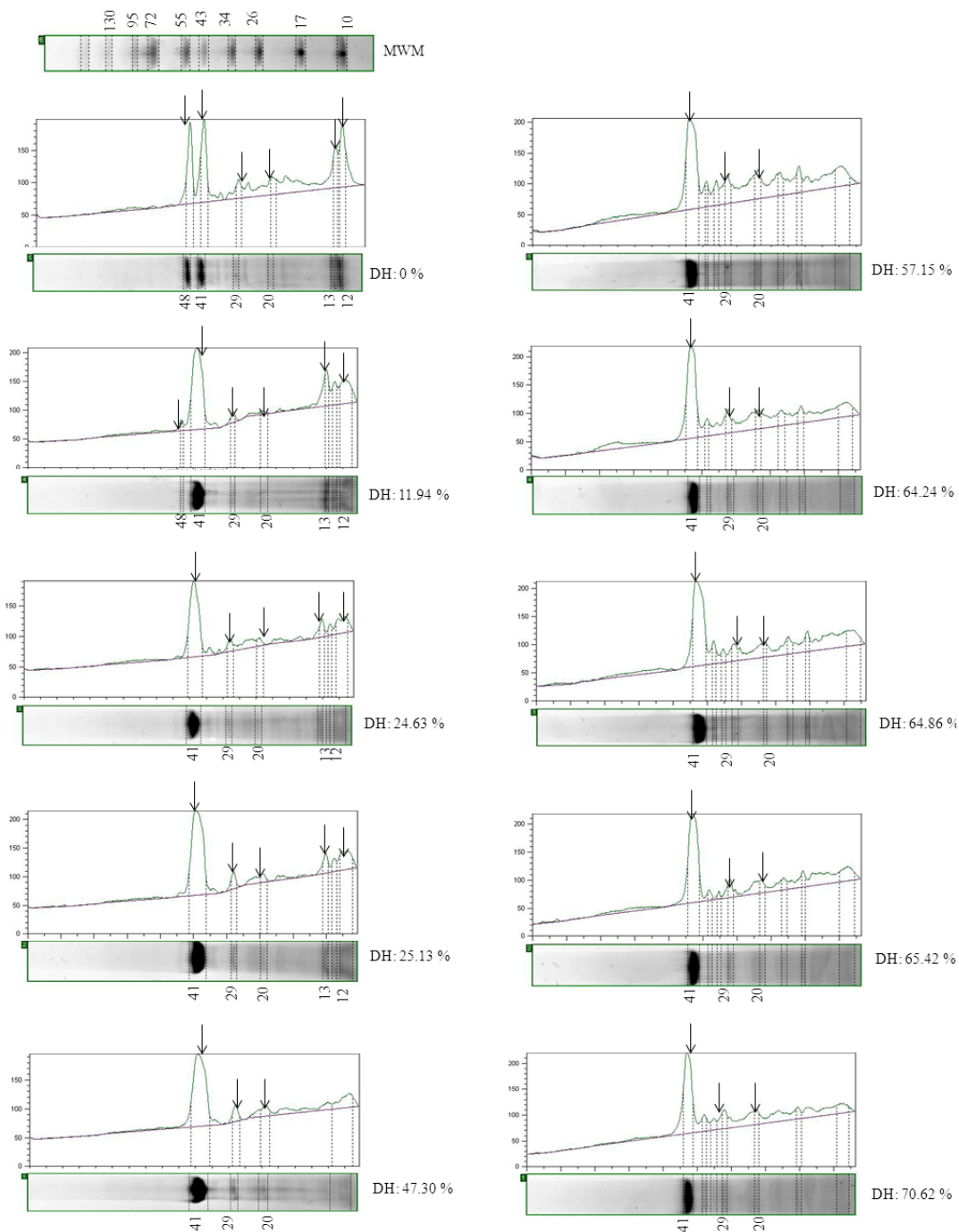


Figure 4.30 Electropherogram images (X axis: Pixel position; Y axis: Pixel intensity) of flaxseed protein isolate and residues remaining after hydrolysis with Flavourzyme (MWM: Molecular weight markers; DH: Degree of hydrolysis)

The precursor proteins responsible for generating peptides with the highest OH[•] scavenging activity as well as antithrombotic activity could be the protein around 48 kDa and the protein around 12 and 13 kDa, as the intensity of these bands were reduced at 24.63 % DH (Figures 4.29 and 4.30). There was complete hydrolysis of polypeptide bands around 48 kDa and 12 kDa at DH of 64.86 % (Figures 4.29 and 4.30; Table 4.30) suggesting that these peptides could be the precursors for generation of O₂^{•-} scavenging peptides.

4.3.4 Optimization of degree of hydrolysis and bioactivities

Analysis of data using response surface methodology (RSM) resulted in the identification of the optimum reaction conditions that produce maximum DH and bioactivities (ACEI, OH[•] and O₂^{•-} scavenging and inhibition of linoleic acid oxidation) in FPHs. Based on the results, the effect of E/S (variable: X₁) and time of hydrolysis (variable: X₂) as well as interactions [e.g. E/S* time of hydrolysis (X₁*X₂)] on the DH, ACEI, OH[•] and O₂^{•-} scavenging and inhibition of linoleic acid oxidation (dependent variables) (See Table 3.1 under Materials and Methodology chapter of this thesis) in the process of hydrolysis were investigated.

Response surface methodology is a statistical technique for designing experiments, building models, evaluating the effects of several factors, and searching for optimum conditions for desirable responses and reducing the number of experiments (Draper & Lin, 1990). In RSM, fewer experimental points can be utilized to evaluate multiple parameters and their interactions, compared to full factor factorial designs thus making it less laborious and time consuming (Cheison *et al.*, 2007). Therefore, the experimental design used in the present study enabled the estimation of the effect of E/S and time of hydrolysis on response variables (DH and bioactivities) and their optimization with minimum experimentation. A quadratic response surface function was estimated by least-squares regression. The predicted optimal values for each of the independent variable were from the estimated response surface. The predictive capabilities of response surface models were explained using the coefficient of determination (R²). The

canonical analysis, which predicts the shape of the response surface curve generated by the model, was also carried out.

4.3.4.1 Optimization of degree of hydrolysis

Figure 4.31 shows the response surface plot obtained for DH in FPHs with the change in time of hydrolysis and E/S. The regression equation (eq 4.1) for DH was obtained after the analysis of variance (ANOVA).

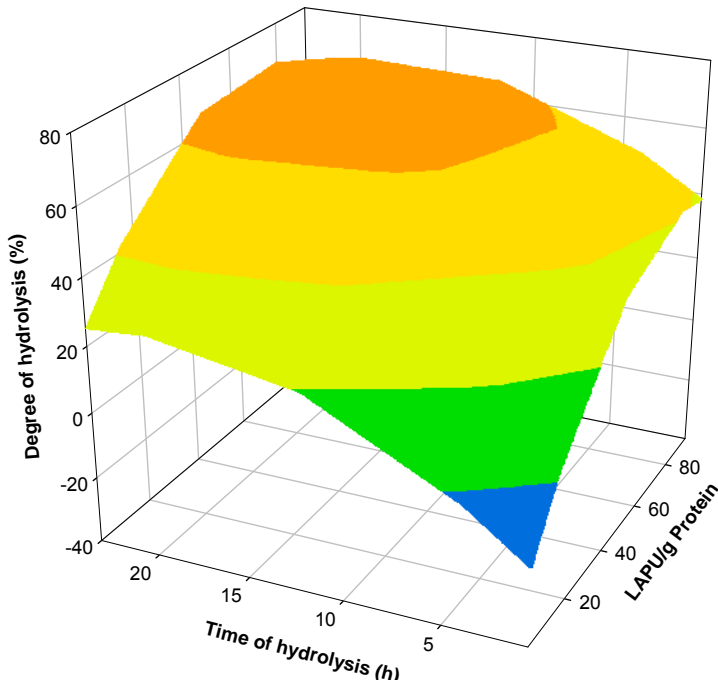


Figure 4.31 Response surface plot for degree of hydrolysis (Y_1) as a function of enzyme to substrate ratio, LAPU/ g of protein (X_1) and time of hydrolysis, h (X_2)

$$Y_1 = -22.42 + 1.58 * X_1 + 4.83 * X_2 - 0.12 * X_2^2 - 0.01 X_1 * X_2 - 0.01 X_1^2 \quad (4.1)$$

The positive coefficients for independent variables (eq 4.1) showed that the increase in both the E/S and time of hydrolysis resulted in an increase in DH. At higher enzyme concentrations, more enzyme catalytic sites are available to participate in the hydrolysis of the substrate, resulting in greater cleavage of peptide bonds (Kurozawa *et al.*, 2008).

Increase in hydrolysis time facilitates the hydrolysis further by improving the contact between enzyme and substrate.

According to the *t* –test and *p* values (results not shown) time, time*time and E/S*time had a significant effect ($P<0.05$) on DH. The regression coefficient (R^2) for the suitability of the response surface model (fit of the model) was 0.92. This indicated that 92 % of the variability for DH can be explained by the independent variables and their interaction components of the model eq 4.1. The high R^2 value indicated that the model was well adapted to the responses. The three-dimensional response surface plot (Figure 4.31) was obtained from the statistically significant ($P<0.05$) model to determine the optimum levels of E/S and time of hydrolysis that yields maximum DH. As shown in Figure 4.31, the DH increased until time of hydrolysis and E/S reached optimum points. The response surface plot had a convex shape indicating a maximum point of DH (Figure 4.31).

The canonical analysis, which is a type of linear statistical analysis used to locate the stationary point (Montgomery, 1991), showed that DH becomes maximum (73.3 %) at the combination of uncoded levels of 69.3 LAPU/ g protein (E/S) and 16.9 h (time of hydrolysis) (Table 4.31).

Table 4.31 Canonical analysis of the response surface for degree of hydrolysis

Response variable	Critical values of hydrolysis factors		Predicted value	Stationary Point
	X_1	X_2		
	E/S ratio	Time (h)		
	(LAPU/g protein)			
DH (%)	69.3	16.9	73.3	Maximum

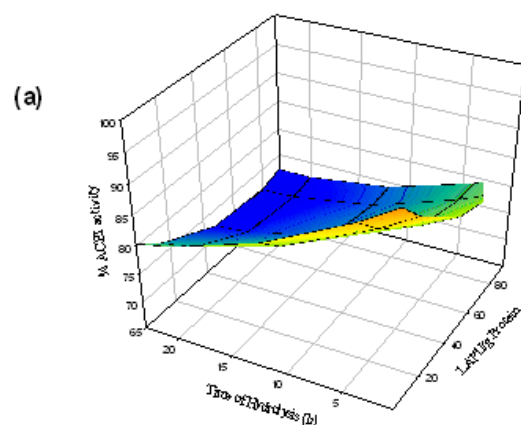
E/S: Enzyme to substrate ratio; LAPU: leucine amino peptidase units
DH: Degree of hydrolysis

4.3.4.2 Optimization of bioactivities

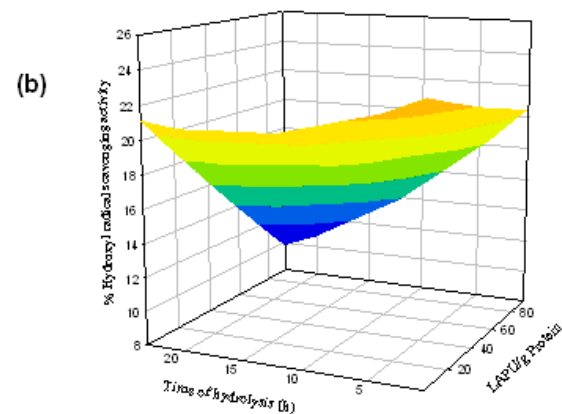
The optimization was carried out for bioactivities such as ACEI activity and antioxidant activities (OH^\bullet scavenging, $\text{O}_2^{\bullet-}$ scavenging, inhibition of linoleic acid oxidation). The response surface plots and their best explanatory equations for bioactivities are given in Figure 4.32. The R^2 of the response surface models for ACEI activity, OH^\bullet scavenging, $\text{O}_2^{\bullet-}$ scavenging activities and inhibition of linoleic acid oxidation were 0.97, 0.83, 0.72 and 0.41, respectively.

The high R^2 value (0.97) of ACEI activity indicated that the model fits well to the data. The response surface plot for ACE inhibition (Figure 4.32a) was generated based on this model. The equation for the model (Figure 4.32a) indicated a decrease in ACEI activity with the increase in E/S and time of hydrolysis in the experimental region and this effect was significant ($P < 0.05$). The positive effect shown by E/S* time on the ACEI activity was found to be not significant ($P < 0.05$). The results confirmed the relationship shown by Figure 4.19, which showed a decrease in ACEI activity with an increase in DH. The response surface had a shape of a valley (Figure 4.32a) and there was no maximum point detected. The stationary point of the surface plot was a minimum, which demonstrated a minimum ACE inhibition (71.2 %) when E/S was 83.7 LAPU/g proteins and time of hydrolysis was 19.9 h (Table 4.32). The optimum conditions (time and E/S) for maximum ACEI activity could not be located by using the model designed for the experiment. Therefore, it can be suggested that higher ACE inhibition could be obtained by minimal hydrolysis of flaxseed protein by Flavourzyme, which will generate lower levels of DH.

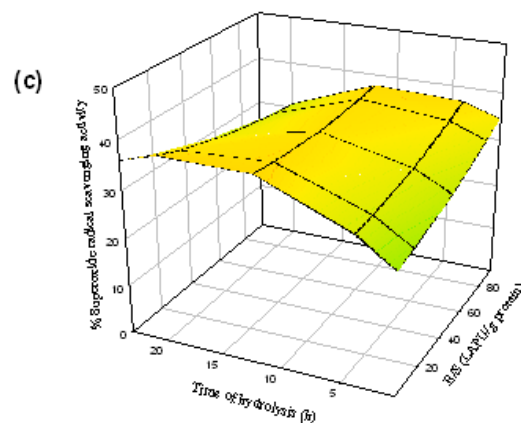
The R^2 of models obtained for antioxidant activities (OH^\bullet scavenging, $\text{O}_2^{\bullet-}$ scavenging, inhibition of linoleic acid oxidation) were lower (R^2 : 0.83 and 0.72, respectively) than that of the models obtained for % ACE inhibition (R^2 : 0.97). However, as shown by the R^2 values, a considerable % of variability in OH^\bullet and $\text{O}_2^{\bullet-}$ scavenging activity (83 % and 72 %, respectively) were explained by the model equations (Figures 4.32b and c). The regression coefficients for both E/S and time of hydrolysis shown by the model equation for OH^\bullet scavenging were negative and they were positive for $\text{O}_2^{\bullet-}$ scavenging activity.



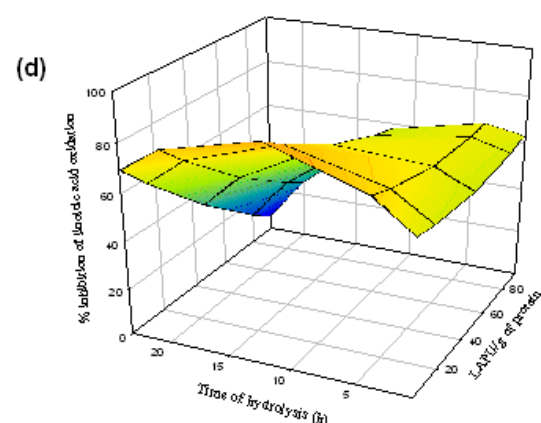
$$Y = 99.09 - 0.37X_1 - 1.26X_2 + 0.002X_1^2 + 0.02X_2^2 + 0.004X_1X_2$$



$$Y = 23.83 - 0.04X_1 - 0.34X_2 + 0.01X_2^2 + 0.0001X_1^2 - 0.004X_1X_2$$



$$Y = 20.61 + 0.09X_1 + 2.09X_2 + 0.0003X_1^2 - 0.06X_2^2 - 0.009X_1X_2$$



$$Y = 60.96 - 0.18X_1 + 4.61X_2 + 0.002X_1^2 - 0.18X_2^2 - 0.03X_1X_2$$

Figure 4.32 Response surface plots for (a) angiotensin I-converting enzyme inhibitory, (b) hydroxyl radical scavenging, (c) superoxide radical scavenging activities and (d) inhibition of linoleic acid oxidation (For X_1 and X_2 refer Tables 4.32 and 4.33)

Table 4.32 Canonical analysis of response surface for angiotensin I-converting enzyme inhibitory activity

Response variable	Critical values of hydrolysis factors		Predicted value	Stationary Point
	X ₁ E/S ratio (LAPU/g protein)	X ₂ Time (h)		
% ACEI activity	83.7	19.9	71.2	Minimum

E/S: Enzyme to substrate ratio; LAPU: Leucine aminopeptidase units

ACEI: Angiotensin I-converting enzyme inhibitory

Therefore, increase in both parameters had a decreasing effect on the ability to scavenge OH[•] but led to an increase in O₂^{•-} scavenging activity. The E/S* time had a negative effect on both antioxidant activities. For OH[•] activity, none of these parameters including their interactions had a significant effect ($P < 0.05$). But for O₂^{•-} scavenging activity, time and the interaction between E/S and time had a significant effect ($P < 0.05$).

The model equation for inhibition of linoleic acid oxidation (Figure 4.32d) explained only 41 % of the variability. An increase in E/S had a negative effect whereas increase in time of hydrolysis had a positive effect on the ability to inhibit linoleic acid oxidation. Also the interaction between E/S and time of hydrolysis had a decreasing effect on the ability to inhibit linoleic acid oxidation (Figure 4.32d). However, none of these effects were significant ($P < 0.05$).

Unlike for ACEI activity, the canonical analysis revealed that the predicted response surfaces for all screened antioxidant activities had saddle points as stationary point (Table 4.33). Hence the estimated surfaces (Figure 4.32) did not have a maximum or minimum point. This indicated that the predicted optimum was away from the region of experiment (Liu *et al.*, 2000). Therefore, a ridge analysis was carried out to locate the critical levels of independent variables that can produce a maximum response (Table 4.33). The ridge analysis indicated that maximum antioxidant activities could be obtained when flaxseed proteins were hydrolyzed for relatively low DH using a moderate level of E/S and time (Table 4.33). The maximum OH[•] scavenging activity

(estimated value: 21.9 %) could be obtained when flaxseed protein was hydrolyzed for 1.5 h at an E/S of 30.2 LAPU/g protein. The ridge analysis also indicated that a maximum $O_2^{\bullet-}$ scavenging (estimated value of 38.6 %) can be obtained when flaxseed protein is hydrolyzed by Flavourzyme at an E/S of 4.9 LAPU/g protein for 16.3 h. Maximum ability for inhibition of linoleic acid oxidation should result when flaxseed protein is hydrolyzed at low E/S (1.6 LAPU/g protein) for 12.6 h.

Table 4.33 Ridge analysis of response surface for antioxidant activities

Response variable	Critical values of hydrolysis factors		Predicted value	Stationary Point
	X ₁ E/S ratio (LAPU/g rotein)	X ₂ Time (h)		
% OH [•] scavenging activity	30.2	1.5	21.9	Saddle
% O ₂ ^{•-} scavenging activity	4.9	16.3	38.6	Saddle
% inhibition of linoleic acid oxidation	1.6	12.6	90.1	Saddle

E/S: Enzyme to substrate ratio; LAPU: Leucine aminopeptidase units
OH[•]: Hydroxyl radical; O₂^{•-}: Superoxide radical

4.3.5 Characterization of bioactive peptides in the most bioactive flaxseed protein hydrolysates

Based on the results, two bioactivities were selected for further study of Flavourzyme hydrolyzed flaxseed protein. They were ACEI and OH[•] scavenging activities. These two bioactivities were also studied on the GI digests of flaxseed protein. The two FPHs that possessed maximum ACE inhibition (DH: 11.94 %) and OH[•] scavenging activity (DH: 24.63 %) were selected for characterization of the bioactive peptides in the FPHs.

4.3.5.1 Amino acid composition of the most bioactive flaxseed protein hydrolysates

The results of amino acid analysis in the most bioactive hydrolysates are given in Table 4.34. Except for Glu, Pro and Val all the other amino acids were greater in the hydrolysate with a DH of 24.6 % (ACEI hydrolysate) compared to that having a DH of 11.94 % (OH[•] scavenging hydrolysate). This was expected as with the increase in DH more peptides and amino acids are released from the protein.

Table 4.34 Amino acid composition (g/100g protein) of the most bioactive hydrolysates

Amino acid	DH (%)	
	11.94 ¹	24.6 ²
<u>Essential</u>		
His	1.59	1.67
Ile ³	3.58	3.78
Leu ³	4.91	5.03
Lys	2.20	2.41
Met	1.98	2.41
Phe	4.63	4.84
Thr	3.15	3.21
Trp	0.89	1.01
Val ³	4.41	4.13
<u>Non-essential</u>		
Ala	3.81	4.29
Arg ⁴	9.75	10.36
Asp	10.03	10.32
Cys	2.26	2.27
Glu	21.13	21.03
Gly	5.23	5.78
Pro	2.69	2.64
Ser	4.77	4.81
Tyr	1.71	1.77
Lys/Arg	0.22	0.23

¹ Hydolysate with the highest ACE inhibition; ² Hydrolysate with the highest OH[•] scavenging activity

³ Branched chain amino acids; ⁴ Conditionally essential

As discussed previously, there were no significant differences ($P<0.05$) between both hydrolysates for ACEI activity and OH[•] scavenging activity. This could be a reason for the absence of any marked differences in amino acid composition between the two hydrolysates. The amino acid composition of these Flavourzyme hydrolyzed flaxseed protein was compared with the hydrolysates of flaxseed protein reported by Udenigwe and Aluko (2010). The authors had calculated Fischer ratios (the ratio between branched chain amino acids and aromatic amino acids) for the flaxseed protein hydrolysates. Protein hydrolysates with higher Fischer ratio are important in the treatment of liver diseases. BCAAs have therapeutic effect as they are reported to be preferentially taken up by muscles, inhibit the transport of aromatic amino acids across blood brain barrier and are available for peripheral metabolism in advanced liver diseases (Clementte, 2000). According to Udenigwe and Aluko (2010), the hydrolysates prepared by treating Thermolysin hydrolyzed flaxseed protein with papain, ficin, Alcalase and pronase had Fischer ratios of 1.84, 1.60, 1.67 and 1.72. The Thermolysin-pronase hydrolysate with the highest Fischer ratio had the ability to be converted to a BCAA rich mixture with a Fischer ratio of 23.65 when mixed with activated carbon.

Based on the findings of the current study, the Fischer ratios of the two hydrolysates with DH of 11.94 % and 24.6 % were 1.78 and 1.69, respectively (Table 4.34). The hydrolysate with 11.94 % DH had a Fischer ratio greater than the highest Fischer ratio (1.72) reported for Thermolysin-pronase hydrolysate of flaxseed protein (Udenigwe & Aluko, 2010). The ratio between Lys:Arg was found to be 0.22 and 0.23 in the hydrolysates with DH values of 11.94 and 24.6 %, respectively (Table 4.34). High Lys:Arg ratios in the diet are reported to be responsible for atherogenicity (Kritchevsky *et al.*, 1982). The Lys:Arg ratios of both FPHs were much lower than that previously reported for cholesterol lowering fish protein hydrolysate (1.1) by Wergedahl *et al.* (2004). This further indicated the potential cardioprotective effect of the selected FPHs.

4.3.5.2 Purification of bioactive peptides

4.3.5.2.1 Purification of angiotensin I-converting enzyme inhibitory peptides using angiotensin I-converting enzyme immobilized to glyoxyl agarose

ACE was immobilized to glyoxyl-agarose and the immobilized ACE was incubated with the FPH possessing maximum ACE inhibition as discussed in the methodology section. Since Captopril (D-3-mercapto-2-methyl-propionyl-L-proline) is a strong competitive ACE inhibitor (Wei *et al.*, 1992) it was hypothesized that Captopril can release any ACEI peptides bound to ACE. The stock solution of commercial ACE used for this study had an activity of 0.14 units/mL. The ACE activity of immobilized ACE was 0.08 units /mL. There was a 33.5 % loss in ACE activity when immobilized ACE was incubated with FPH showing the highest ACEI activity. This reduction in ACE activity was expected to be the result of binding ACEI peptides in the FPH with the active site of ACE. The FPH recovered after incubating with immobilized ACE was expected to have a size exclusion chromatogram different from the original FPH due to binding of peptides with ACE. Although a reduction or absence of a peptide peak in the FPH was expected, no clear difference was detected between the chromatograms of the FPH before and after incubation (Figure 4.33).

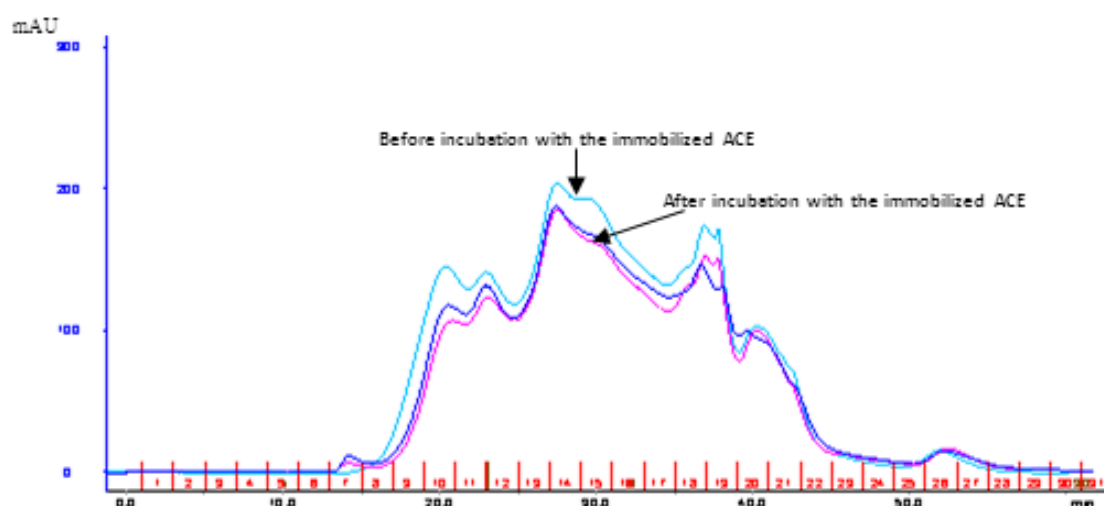


Figure 4.33 The size exclusion chromatograph of flaxseed protein hydrolysate (Degree of hydrolysis=11.9 %) before and after incubation with immobilized angiotensin I-converting enzyme. The absorbance was monitored at 214 nm

ACE immobilized to glyoxyl-agarose have been successfully used to purify ACEI peptides in sunflower protein hydrolysates prepared by sequential hydrolysis using Alcalase and Flavourzyme (Megias *et al.*, 2009) as well as Alcalase hydrolyzed rapeseed protein (Megias *et al.*, 2006). As commercial ACE is expensive this method was also expected to produce immobilized ACE that can be reused in ACE assays. However, in our study this was not possible due to the inability to detect the binding of ACEI peptide with immobilized ACE. Therefore, an alternative method was explored for the purification of the ACEI peptides by peptide fractionation using size exclusion column (SEC) connected to AKTA FPLC system followed by further concentration of peptides with RP-C18 spin columns. The same purification was carried out for FPH with high OH[•] Scavenging activity.

4.3.5.2.2 Peptide fractionation of flaxseed protein hydrolysates

The fractionation of FPHs was carried out using SEC connected to an AKTA FPLC. Figure 4.34 shows the molecular mass distribution profile of the FPHs that showed the highest levels of the selected bioactivities. Five peptide fractions namely F-I (>2.5 kDa), F-II (1.05-2.5 kDa), F-III (0.5-1.05 kDa), F-IV (<0.5 kDa) and F-V (<0.5 kDa), were collected from each FPH based on the molecular mass (Figure 4.32).

As shown by the absorbance at 280 nm, F-I, F-III, F-IV and F-V of all the FPH had phenolic groups or aromatic amino acid containing peptides (Figure 4.34). The peptide fractions obtained from each FPH were screened for the respective bioactivities shown by the unfractionated FPH. The results are displayed in Table 4.35. Amongst the fractions collected from the FPH having the highest ACEI activity, (Fig.4.34 a), F-I (>2.5 kDa) had no ACEI activity. The F-III (0.5 to 1.05 kDa) fraction had significantly higher ($P<0.05$) ACEI activity (76.7 ± 1.2 %) compared to other fractions. Therefore F-III was identified to contribute most to the ACEI activity of the FPH with DH of 11.94 % followed by F-IV, F-II and F-V. The IC₅₀ for ACEI activity was calculated only for F-III as it had the highest ACE inhibition. Also the peptides in F-III were expected to be mostly short chain peptides (<1 kDa).

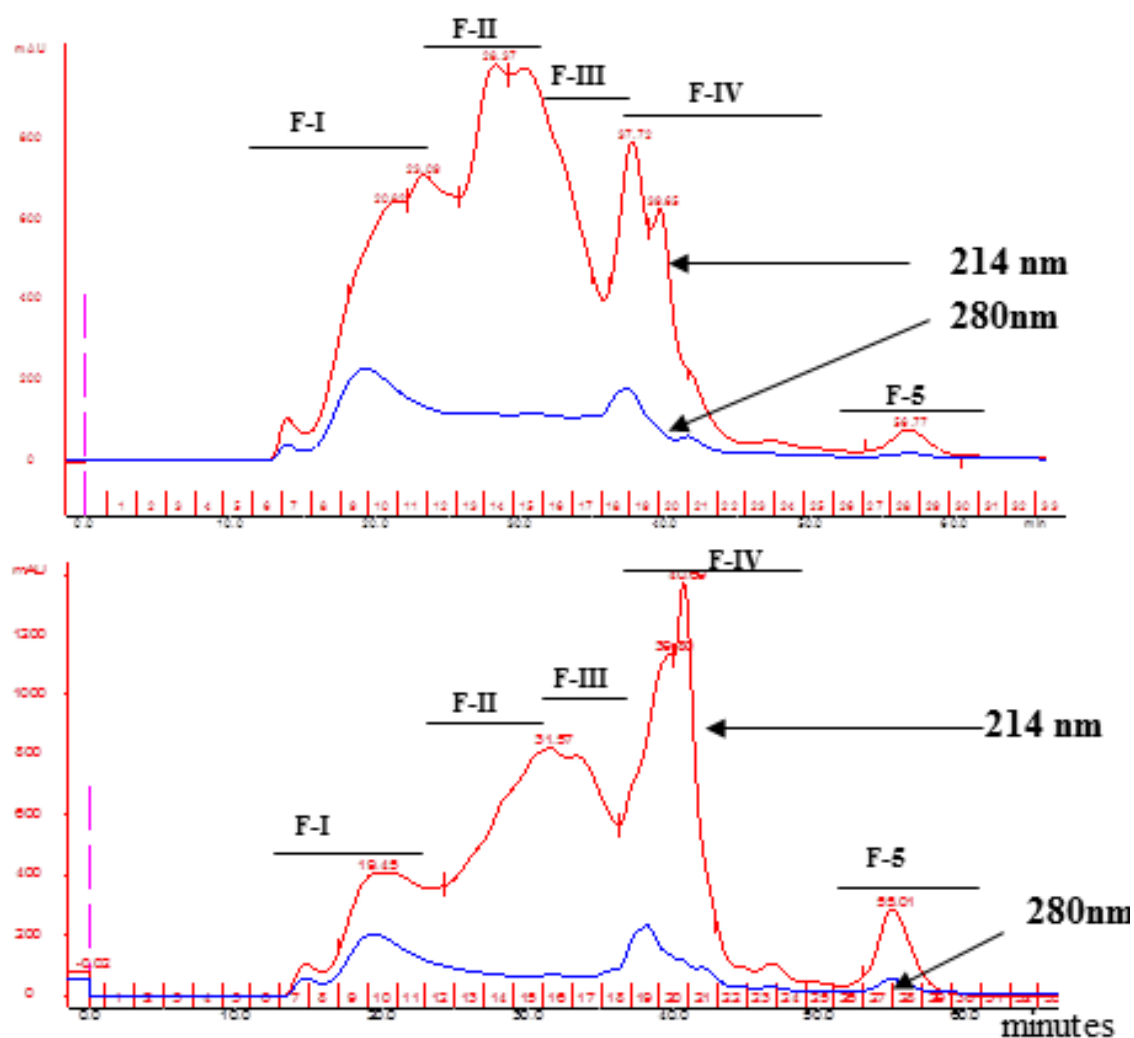


Figure 4.34 Elution profiles of flaxseed protein hydrolysates [(a) Angiotensin I-converting enzyme inhibitory activity: 88.29 %; Degree of hydrolysis: 11.94 %, (b) Hydroxyl radical scavenging activity: 22.08 %; Degree of hydrolysis: 24.63 %] separated by FPLC using a size exclusion column (Superdex 10/30). The column was equilibrated and eluted with 20 % (v/v) Acetonitrile having 0.05 % (v/v) TFA, at a flow rate of 0.5 mL/min

Table 4.35 Bioactivities of peptide fractions of flaxseed protein hydrolysates in relation to molecular mass distribution

Fraction	Molecular mass distribution of peptides in the fraction (kDa)	Flaxseed protein hydrolysates			
		DH: 11.94 %		DH: 24.63%	
		ACEI activity (%) ^{1,2}	IC ₅₀ (mg/mL) ¹	OH [•] scavenging activity (%) ^{1,3}	IC ₅₀ (mg/mL) ¹
F-I	>2.5	0 ^c	N.D.	43.39 ± 0.0 ^a	N.D.
F-II	1.05-2.5	27.5 ± 1.1 ^b	N.D.	24.39 ± 0.4 ^b	N.D.
F-III	0.5-1.05	76.7 ± 1.2 ^a	0.08 ± 0.0	43.45 ± 4.5 ^a	0.51 ± 0.0
F-IV	<0.5	38.2 ± 0.5 ^b	N.D.	22.96 ± 1.1 ^b	N.D.
F-V	<0.5	1.6 0 ± 1.3 ^c	N.D.	0 ^c	N.D.

¹Values are means ± standard deviations of duplicate analyses

^{abc}Means in the same column followed by same letter are not significantly different ($P < 0.05$)

²Concentration used: 0.3 mg/mL; ³Concentration used: 0.5 mg/mL

DH: Degree of hydrolysis; ACEI: Angiotensin 1-converting enzyme inhibitory; OH[•]: Hydroxyl radical
N.D.: Not determined

The interest in short chain peptides was due to reported evidence that they are able to withstand proteolytic degradation by the enzymes in GI tract when orally ingested by humans (Aluko, 2008) and the ability to be absorbed without further hydrolysis. The IC₅₀ for F-III was 0.08 mg/mL (Table 4.35). This was higher than the ACEI IC₅₀ shown by the unfractionated FPH (0.07 mg/mL). Therefore, F-III had a weaker ACEI potency than the unfractionated FPH. The possible reason could be the synergistic effect on ACEI activity by different peptides in the hydrolysate. Therefore, separation of peptides according to the size resulted in a loss of ACEI activity, which might be because of the separation of synergistically active peptides. However, this F-III had higher ACEI potency than the strongly cationic <1 kDa fraction of Alcalase hydrolyzed flaxseed protein (IC₅₀: 0.12 mg/mL) reported by Udenigwe *et al.* (2009). The ACEI IC₅₀ in F-III was greater than the <1 kDa fraction of Thermolysin hydrolyzed flaxseed protein (0.02 mg/mL) and weakly cationic <1 kDa fraction of Alcalase hydrolyzed flaxseed protein (~0.03 mg/mL) reported by Udenigwe *et al.* (2009). However, it the values reported by Udenigwe *et al.* (2009) were for <1 kDa fraction. Hence, the IC₅₀ reported by Udenigwe

et al. (2009) could be the result of synergistic effects of many LMW peptides, whereas in the current study the IC₅₀ was analyzed only for the 0.5-1.05 kDa fraction. Therefore, the contribution by peptides less than 0.5 kDa were not included. The absorbance at 280 nm suggested the presence of phenolic groups or aromatic amino acids of the peptides in F-III indicating that the ACEI peptides in this fraction could possess hydrophobic side chains. This is in accordance to Murray and FitzGerald (2007) and Cheung *et al.* (1980) who reported that Pro and aromatic amino acids play an important role in ACEI peptides for binding with ACE

The FPH with 24.6 % DH had the highest OH[•] scavenging activity (22.1 %) (Table 4.27). The F- I (>2.5 kDa) and F-III (0.5 to 1.05 kDa) of this FPH had superior OH[•] scavenging activity (43.39 ± 0.0 % and 43.45 ± 4.5 %, respectively) compared to that of the unfractionated FPH (Table 4.27) when used at the same concentration. The OH[•] scavenging activity shown by F-I and F-III of the hydrolysate (DH: 24.63 %) were significantly higher (*P*<0.05) than those of other fractions (Table 4.35). However, there was no significant difference between F-I and F-III of the hydrolysate (DH: 24.63 %) for OH[•] scavenging activity. In contrast, according to Udenigwe *et al.* (2009), the HMW fraction (>1 kDa) of Alcalase hydrolyzed flaxseed protein did not show OH[•] scavenging activity. In the current study, IC₅₀ for OH[•] scavenging activity was only calculated for F-III as it had high activity with LMW peptides (0.5 to 1.05 kDa) that have potential for intestinal absorption. The IC₅₀ (0.51 mg/mL) of this fraction was much lower and therefore its OH[•] scavenging potency was greater than that of the unfractionated FPH (1.56 mg/mL). The presence of both pro-oxidants and antioxidants, could be the reason for the lower OH[•] scavenging activity in the unfractionated FPH. Removal of the associated pro-oxidants during the fractionation process could have enhanced this activity in the fractions. The F-III fraction obtained from the hydrolysates with DH 11.94 % and 24.63 % (showing high ACEI activity and OH[•] scavenging activity, respectively) were selected for further characterization of bioactive peptides.

4.3.5.3 Amino acid sequence of peptides in the most bioactive flaxseed protein hydrolysate fractions

Table 4.36 shows the amino acid sequence of peptides identified in the most bioactive FPLC fraction of the hydrolysates, which had the highest ACEI and OH[•] scavenging activities.

Table 4.36 Amino acid sequence of peptides in the most bioactive fractions of flaxseed protein hydrolysates deduced from *de novo* sequencing

DH (%)	Bioactivity	FPLC fraction	Amino acid sequence of peptides	Molecular mass	Probability ¹
11.94	ACEI activity	F-III (0.5-1.05 kDa)	Gly-Leu-Leu-Leu-Pro-Phe (GLLLPF)	658.41	37.29
			Val-Val-His-Val-Leu-Leu (VVHVLL)	678.44	21.14
24.63	OH [•] scavenging activity	F-III (0.5-1.05 kDa)	Asp-Val-Ala-Leu-Leu-Pro-Ala (DVALLPA)	697.40	59.11
			Phe-Thr-Pro-Met-Pro-Glu (FTPMPE)	720.19	74.23
			Pro-Ser-Asp-Glu-Gln-Phe (PSDEQF)	721.19	88.17
			Met-Pro-Leu-Asp-Val-Leu-Ser(MPLDVLS)	773.24	100.00
			Trp-Pro-Leu-Asp-Glu (WPLDE)	658.26	76.19

¹Obtained using the MassLynx software
FPLC: Fast Protein Liquid Chromatography
The single letter coding for amino acids are given within paranthesis

The two proposed peptide sequences in the most ACEI fraction of the flaxseed protein hydrolysate had relatively lower probability compared to that of the other peptides. Both these peptides had 6 amino acid residues. Similar to peptides obtained from GI digestion, none of these two peptide sequences matched with the amino acid sequence with conlinnin, the LMW flaxseed storage protein with a known amino acid sequence. Therefore, it is likely that these ACEI peptides are not derived from the sequence

known protein of flaxseed. The role of different amino acids in the peptides for its ACEI activity was discussed in detail under section 4.1.5.5. of the results and discussion chapter in this thesis. The importance of C-terminal aromatic amino acids (Trp, Tyr, Phe) and Leu for ACE inhibition has been shown by previous studies (Cheung, 1980; Gomez-Ruiz *et al.*, 2007). Presence of Phe and Leu in the C-terminus of identified peptides could contribute to the ACEI potency of the identified peptides. Interestingly, Pro was in the C-terminal penultimate position in the peptide, Gly-Leu-Leu-Leu-**Pro**-Phe, which is reported to induce low affinity of the inhibitor towards ACE (Cheung *et al.*, 1980). Since the chain length of the identified peptides include more than 2-3 amino acid residues, it is possible that the steric effects of the peptide may be masking any negative influence caused by Pro in binding with ACE.

Amino acid sequence of five peptides were proposed for the hydrolysate fraction with the highest OH[•] scavenging activity. The chain lengths of the peptides ranged from 5-7 amino acid residues. Similarly, to the peptides in the most ACEI fraction, none of these peptides matched with the sequence in conlinnin. Generally, the scavenging of free radicals was attributed to the donation of hydrogen/electron. Structural features of some amino acids are found to be important for the free radical scavenging activity of peptides.

For example, the imidazole group in His (Chen *et al.*, 1995), indolic group in Trp and phenolic group in Tyr (Hernandez-Ledesma *et al.*, 2005; Guo *et al.*, 2009) act as hydrogen donors. Aromatic amino acids are generally considered as effective radical scavengers. They donate protons easily to free radicals that are electron deficient while maintaining their stability via resonance structures (Rajapakse *et al.*, 2005). Moreover, Pro is reported to be an effective radical scavenger and Met residues also have been reported to contribute to the antioxidant activities of soybean peptides (Chen *et al.*, 1996) and fermented milk peptides (Hernandez-Ledesma *et al.*, 2005). Met is prone to oxidation into Met sulfoxide and Cys donates the sulphur hydrogen (Hernandez-Ledesma *et al.*, 2005). All the peptides identified in the most OH[•] scavenging FPH fraction had Pro residues. The peptides Phe-Thr-pro-Met-Pro-Glu, Pro-Ser-Asp-Glu-

Gln-Phe and Trp-Pro-Leu-Asp-Glu had aromatic amino acids in their sequence whereas Met, was found in two of the identified peptides (Phe-Thr-Pro-Met-Pro-Glu and Met-Pro-Leu-Asp-Val-Leu-Ser), which explained the reason for high antioxidant activity of the hydrolysate fraction.

In addition to the amino acid composition, the role of the position of amino acids and specific amino acid sequences on antioxidant activity of peptides has been identified. Chen *et al.* (1998) reported that peptides containing His at the C-terminus could act as effective scavengers towards various radical species. Suetsuna *et al.* (2000) found that the radical scavenging activity of the peptide Tyr-Phe-Tyr-Pro-Glu-Leu was strongly dependent on the dipeptide Glu-Leu. In addition, the amino acid sequence of antioxidant peptides such as Pro-His-His in soybean peptide (Saito *et al.*, 2003), Gly-Pro-Hyp in peptides of bovine skin gelatin hydrolysate (Kim *et al.*, 2001) and Gly-Pro in peptide of fish skin gelatin are responsible for radical scavenging. However, none of the above sequences were found in the OH[•] scavenging peptides identified in the present study.

The OH[•] scavenging activity was found in the gastric + intestinal digest obtained from the static model of simulated GI digestion but not in the dynamic model (See results of study I). The results of study I suggested that although flaxseed protein contains OH[•] scavenging peptide sequences, GI digestion might not release these peptides from flaxseed protein. The present study showed that both ACEI and OH[•] scavenging peptides can be released by hydrolyzing flaxseed protein with Flavourzyme. The bioactive FPHs produced by Flavourzyme catalyzed hydrolysis of flaxseed protein could be used as potential functional food ingredients. However, it is important to consider that the bioactive peptides in the FPHs will be exposed to the attack of digestive enzymes in the GI system upon consumption, which will further cleave these peptides either improving or reducing their activity. Therefore, further research is necessary prior to drawing any conclusion regarding the ability of such peptides identified in the present study to exert any health effect.

4.3.5.4 Summary of the findings of study III

The results of the present study indicated that extensive degree of hydrolysis of flaxseed protein (up to a DH of 70 %) could be achieved by Flavourzyme catalyzed hydrolysis. Hydrolysis of flaxseed protein by Flavourzyme resulted FPHs with ACEI and antioxidant activities (scavenging of OH^\bullet and $\text{O}_2^{\bullet-}$, inhibition of linoleic acid oxidation). Flaxseed protein hydrolysate with maximum ACEI activity shows competitive inhibition of ACE. The ACEI and OH^\bullet scavenging activity was greater in FPHs with long average PCL than the short chain peptides. For example maximum ACEI and OH^\bullet scavenging activities were shown by FPHs having DH values of 11.94 % (IC_{50} : 0.07 mg/mL) and 24.63 % (IC_{50} : 1.56 mg/mL), respectively. The peptides ranging between 0.5 kDa to 1.05 kDa in the respective FPHs contributed most to its ACEI activity and OH^\bullet scavenging activity.

The $\text{O}_2^{\bullet-}$ scavenging and the ability to inhibit linoleic acid oxidation did not show any relationship with the DH. The FPHs had poor metal chelating ability and bile acid binding ability. Only a few FPHs showed antithrombotic activity.

The unhydrolyzed flaxseed protein did not possess ACEI activity, OH^\bullet , $\text{O}_2^{\bullet-}$ scavenging activities and antithrombotic activity, but had metal chelating activity and the potential to inhibit linoleic acid. Flavourzyme catalyzed hydrolysis reduced the metal chelating activity of FPHs and improved the ability to inhibit linoleic acid oxidation in most of the FPHs. The unhydrolyzed flaxseed protein had better ability to bind bile acids *in vitro* than the FPHs. As flaxseed protein undergoes further hydrolysis in the GI system the bioactivities shown by the protein will be altered during the digestion process and therefore, was considered as not important.

Of the polypeptides in isolated flaxseed protein, the 48 kDa polypeptide was the potential precursor of generating peptides with ACEI activity and ability to inhibit linoleic acid oxidation. The precursor proteins for OH^\bullet and $\text{O}_2^{\bullet-}$ scavenging peptides were the polypeptides of 48 kDa and 12 kDa.

The optimization study revealed that a maximum DH can be obtained when flaxseed protein is hydrolyzed with Flavourzyme at an E/S of 69.3 LAPU/ g of protein for 16.9 h. The optimal reaction condition for ACEI activity was the hydrolysis of flaxseed protein at an E/S of 83.7 LAPU/ g protein for 19.9 h. For the antioxidant activities, the optimum reaction conditions could not be located in the range of experimental conditions used (E/S and time of hydrolysis) in this study and therefore estimated using ridge analysis. These values were, hydrolysis of flaxseed protein at 30.2 LAPU/g protein for 1.5 h for OH[•] scavenging activity, 4.9 LAPU/g protein for 16.3 h for O₂^{•-} scavenging activity, and 1.6 LAPU/ g protein for 12.6 h for inhibition of linoleic acid oxidation.

De novo sequencing was able to deduce two peptide sequences, Gly-Leu-Leu-Leu-Pro-Phe and Val-Val-His-Val-His-Leu, from the most ACEI fraction (0.5-1 kDa) of the FPH (DH: 11.94 %). Five peptides with amino acid sequences of Asp-Val-Ala-Leu-Leu-Pro-Ala, Phe-Thr-Pro-Met-pro-Glu, Pro-Ser-Asp-Glu-Gln-Phe, Met-pro-Leu-Asp-Val-Leu-Ser, and Trp-Pro-Leu-Asp-Glu were deduced from the most OH[•] scavenging peptide fraction (0.5-1 kDa) of the FPH (DH: 24.63 %).

5. CONCLUSIONS

The findings of the current research provide supportive evidence for cardioprotective health claims for flaxseed and its products, encouraging researchers to switch their focus from ALA, lignan and mucilage towards the protein component in seed. The following major conclusions can be drawn from the studies presented in this thesis.

- Flaxseed proteins consist of ACEI peptide sequences that are released during simulated GI digestion. Several of these peptides of less than 1 kDa molecular mass can pass through the intestinal epithelium via paracellular diffusion implying that flaxseed protein consumption can generate a blood pressure lowering effect via ACEI mechanism of the released bioactive peptides. The antihypertensive effect of flaxseed protein needs to be further confirmed by *in vivo* studies. The highest ACEI potency is found in the peptides of 0.5-1 kDa in the absorbable fraction of GI digested flaxseed protein. The peptides of 5-7 amino acid residues, that are abundantly found in this potent group require structure function confirmation of their ACEI activity. Lack of matching peptide fragments in conlinin (sequence available) to the found ACEI peptides indicates possible involvement of other seed storage proteins such as linin for the release of these peptides. The exact precursor protein in flaxseed that releases ACEI peptides can be fully ascertained once the complete sequence of flaxseed proteome becomes available.
- Of the flaxseed polypeptides, those above 13 kDa (48, 41, 29 and 20 kDa) could be the sources releasing ACEI peptides during GI digestion.
- Although flaxseed proteins contain OH[•] scavenging peptide sequences, release of absorbable peptides with such activity during GI digestion is unlikely.

- Generation of peptides with bioactivities from flaxseed proteins was different when intestinal digestion includes continuous removal of digestion products (<1 kDa) in comparison with a system that did not remove such products. Careful selection of conditions and interpretation of results is needed when generation of bioactive peptides during *in vitro* GI digestion is reported.
- Oil and mucilage in flaxseed reduces flaxseed protein digestibility. The limited digestibility of proteins may limit the potential by whole flaxseed or milled/ground flaxseed to generate ACEI peptides limiting any antihypertensive effect. Defatted meal, which is low in oil, would be a potential source of ACEI peptides upon removal of mucilage.
- Limited hydrolysis of flaxseed proteins with the commercial food grade protease Flavourzyme, releases peptides with ACEI and antioxidant activities. Bile acid binding ability is limited to unhydrolyzed flaxseed protein.
- The 48 kDa and 13 kDa polypeptide bands in flaxseed protein could be the precursors of ACEI and antioxidant peptides released during Flavourzyme catalyzed hydrolysis.
- Hexa- and heptapeptides of 0.5-1.05 kDa, generated from Flavourzyme hydrolysis, are the primary contributors for ACEI and OH[•] scavenging activities. The ability of these peptides to resist GI digestion and retain bioactivities warrant further studies.

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APPENDICES

APPENDIX I

A.1 Simulated GI digestion of flaxseed protein: static model I (with no simulated absorption)

In the static model I, GI digestion conditions to simulate the human digestion system were identified based on the published literature. According to Oomen *et al.* (2003), human digestive juices are secreted at the ratio of 1:1.5:3:1 (Saliva: gastric Juice: Pancreatic juice: Bile). However, simulated saliva with amylase was not used in the current study due to lack of starch in flaxseed. Therefore, the ratio between volumes of digestive juices to be used in the static model I were determined as gastric juice 18 mL: Pancreatic juice 36 mL: Bile 12 mL. The most suitable conditions for simulated GI digestion of flaxseed protein were determined based on the digestibility of casein.

A.1.1 Determination of digestion conditions

A.1.1.1 Determination of amount of protein to be used in the simulated GI digestion study - static model I

Recommended daily protein intake	=0.8 g/kg of body weight/day (Food and Nutrition Board, 2005)
Average weight of male (Canadian)	=83.2 kg (Gilmore, 1999)
Average weight of female (Canadian)	=65.8 kg (Gilmore, 1999)
Recommended protein intake (adult male)	= 83.2×0.8 =66.56 g/day
Recommended protein intake (adult female)	= 65.8×0.8 =52.64 g/day
Average protein intake by the population (To meet recommended intake values)	= $(66.56 + 52.64)/2$ g/day =59.6 g/d
Number of meals taken per day	=3

Amount of protein taken per meal (g) =59.6/3

=19.86 or ~20 g

Due to limitations in the size of reaction flask 1 g of protein was used for the digestion study and the digestive juices were scaled down accordingly.

A.1.1.2 Determination of enzyme to substrate ratios and amount of digestive juices

(a) Pepsin: protein ratio

Armand *et al.* (1995) reported the pepsin output of healthy adults upon receiving a low fat diet, at basal level as well as upon pentagastrin stimulation. The pentagastrin stimulated pepsin output recorded in the study by Armand *et al.* (1995) was used to determine the pepsin in gastric juice of our simulated GI digestion study.

The reported pepsin output =128961 U/h.

The amount of pepsin in collected gastric juice = 718000 U/L

Approximate time food retain in stomach =2 h

Amount of pepsin secreted for 2 h =128961 × 2= 257922 U

Pepsin: Protein ratio =257922 U/20g protein

=12.89 U/mg protein

Amount of pepsin used for 1g of flaxseed protein =12890 U

Volume of gastric juice having 12890 U pepsin =(1000 mL/718000 U)*12890 U

=17.95 mL

(b) Volume of Pancreatic juice and pancreatin: protein ratio

Okefee *et al.* (2006) have reported the Trypsin output after oral diet as 429.5 NFU/h

NFU (National Formulary units) =USP

Time for intestinal digestion =6 h

Trypsin output for 6 h =429.5 × 6

=2577 USP

Trypsin: Protein ratio =2577/20000

=0.13 USP/mg protein

Amount of trypsin for 1g of flaxseed protein= 130 USP

Table A1 digestion conditions used in the static model I

Digestion phase	pH	Time	Digestive juice	Composition	Enzyme	E/S
Gastric	2	2 h	Gastric juice	718000 U of pepsin/L of gastric control solution. (Sigma pepsin:3660 units of pepsin/mg solid)	Pepsin	12.89 U/mg protein
Intestinal	7.5	6 h	Pancreatic juice	3611.1 USP of pancreatic trypsin /L of small intestinal control solution	Pancreatin	130 USP/g protein

A.1.2 Determination of ratios between digestive fluids

The volume of total digestive secretions in man is estimated to be 10 L/day, i.e., around 3 L/meal. (Laurent *et al.*, 2007). Ratio between gastric juice; pancreatic juice: bile is estimated to be 1:1.5:3:1. According to the digestive juice ratios volume of pancreatic juice used will be 35.9 mL. Protease activity of sigma porcine pancreatin according to manufacturer: 209 USP/mg solid for 1 g protein.

Gastric juice 17.95 mL: Pancreatic juice 35.9 mL: Bile 11.96 mL

A.1.3 Preparation of digestive fluids

The gastric control solution and intestinal control solution was prepared according to US Pharmacopeia (1995) with modifications in the pH. The simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared fresh by mixing pepsin and pancreatin with the respective control solutions. The bile solution was prepared fresh by mixing 6 g of porcine bile extract (Sigma) in 1L of intestinal control solution. The mixture was centrifuged at $9820 \times g$ for 15 min and filtered using whatman number 1 filter paper prior to use.

A.1.4 Gastric phase digestion

One gram of casein was suspended in 12 mL deionized water and the pH of the suspension was adjusted to 2 ± 0.1 . The mixture was incubated in a double jacketed glass container at 37 °C under mild stirring for 5 minutes. The gastric phase of the digestion was initiated by adding 18 mL of SGF (100 mL of the gastric fluid contained 0.2 g of NaCl + 71800 U pepsin and HCL was added to adjust the pH to 2) to have a pepsin: protein ratio of 13 U/mg. The digestion was carried out at 37 °C for 2 h in a double jacketed glass container. The contents were stirred using a magnetic stirrer. The gastric phase digestion was terminated by raising the pH to 7.5 using 1 M NaOH.

A.1.5 Intestinal phase digestion

The digest from gastric phase was mixed with 12 mL of bile solution and 36 mL of SIF (E/S: 130 USP of pancreatic trypsin/g protein). The mixture (pH=7.5) was stirred at 37 °C for 4 h.

A.1.6 Collection of samples

Samples were collected at zero time and both at the end of gastric digestion and intestinal digestion. At the end of all phases the digestion was stopped by heating at 98 °C for 15 minutes in a water bath.

APPENDIX II

MS/MS spectra of the most abundant peptides identified in the fraction of gastric digest with the highest ACEI activity

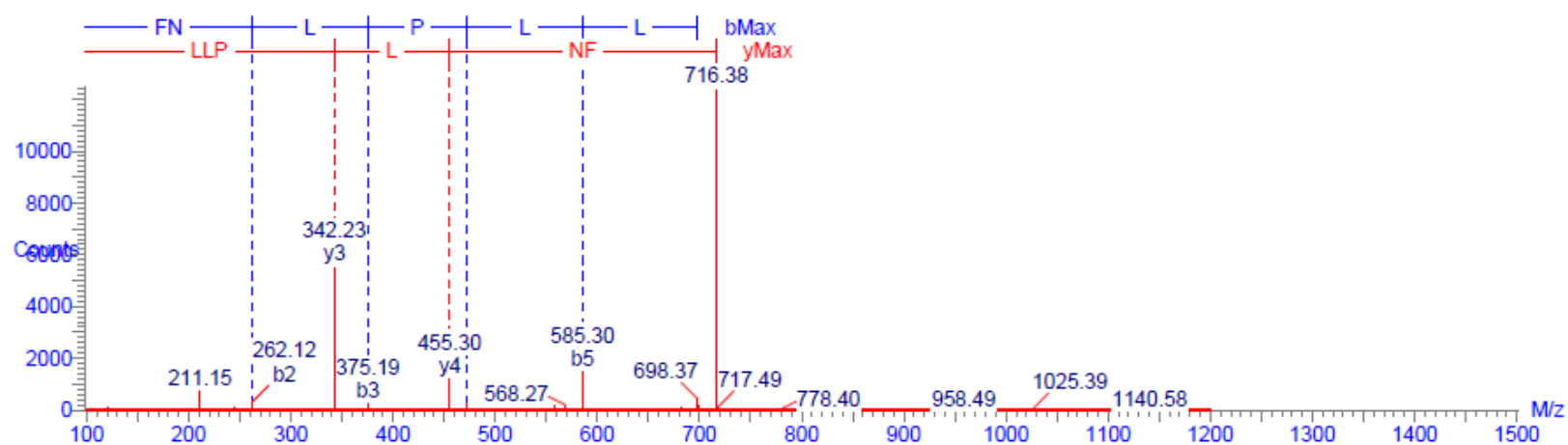


Figure A1. MS/MS spectra of the peptide Phe-Asn-Leu-Pro-Leu-Leu

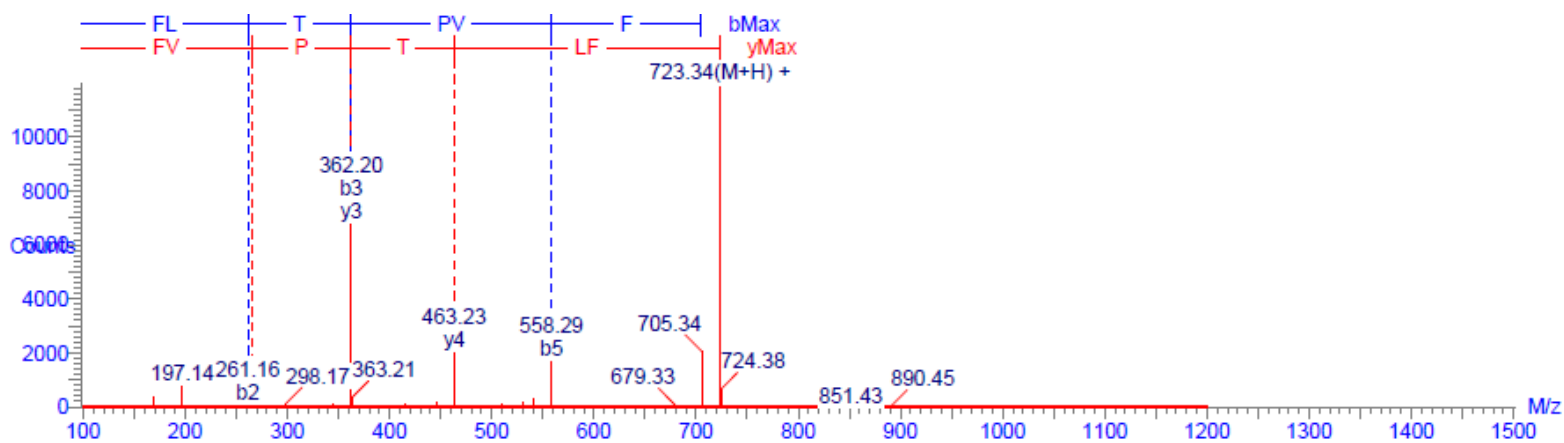


Figure A2. MS/MS spectra of the peptide Phe-Leu-Thr-Pro-Val-Phe

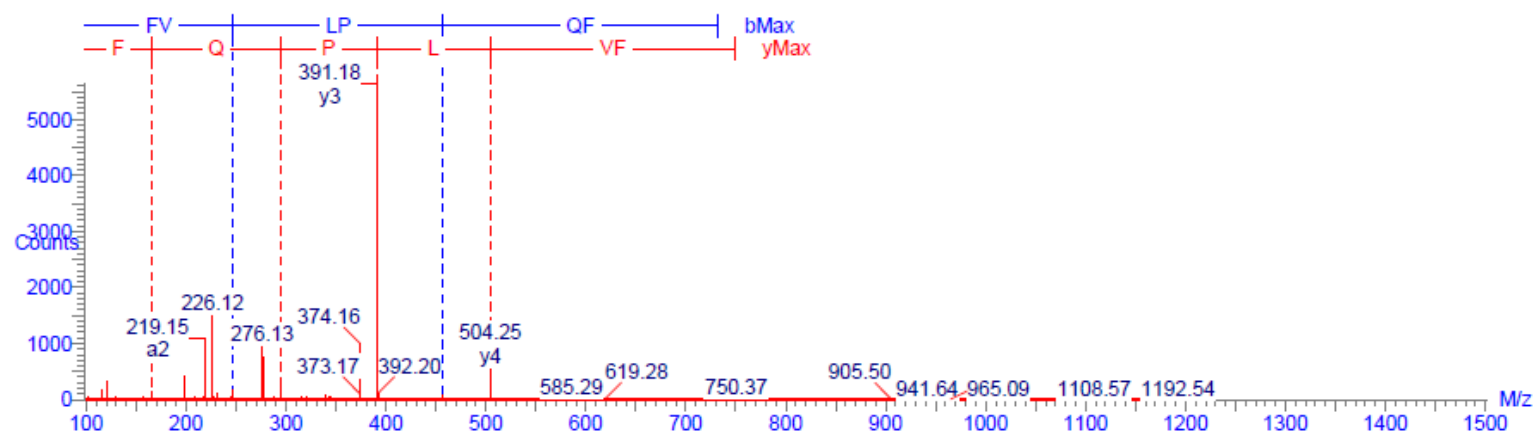


Figure A3. MS/MS spectra of the peptide Phe-Val-Leu-Pro-Gln-Phe

MS/MS spectra of the most abundant peptides identified in the fraction of gastric + intestinal digest with the highest ACEI activity

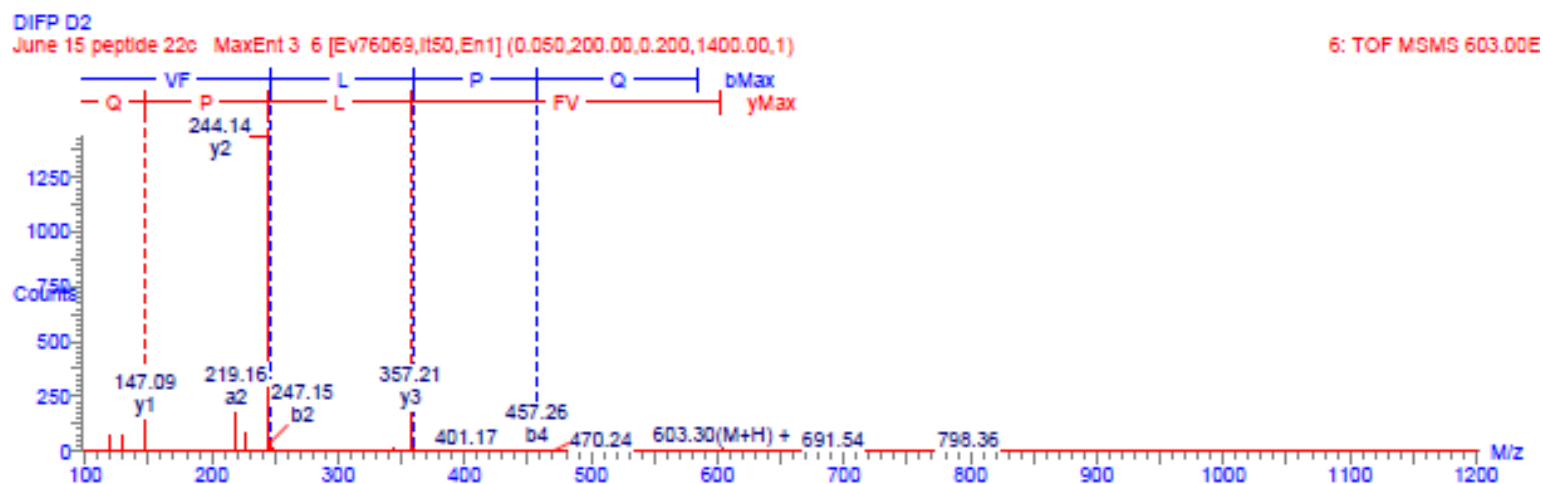


Figure A4. MS/MS spectra of the peptide Val-Phe-Leu-Pro-Gln

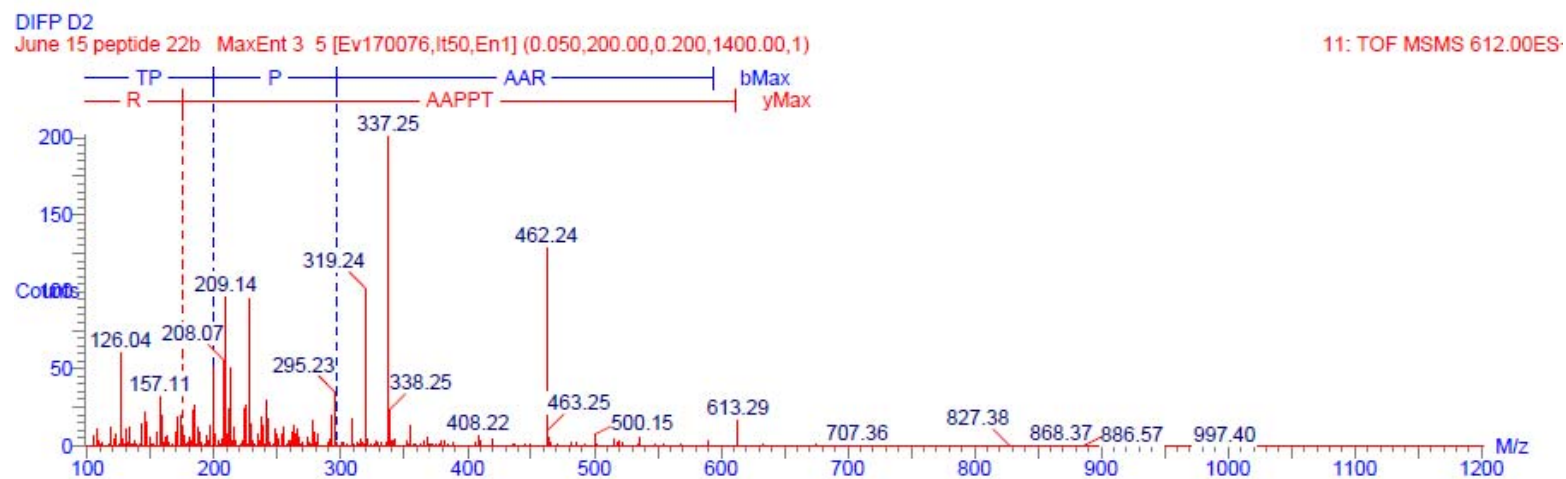


Figure A5. MS/MS spectra of the peptide Thr-Pro-Pro-Ala-Ala-Arg

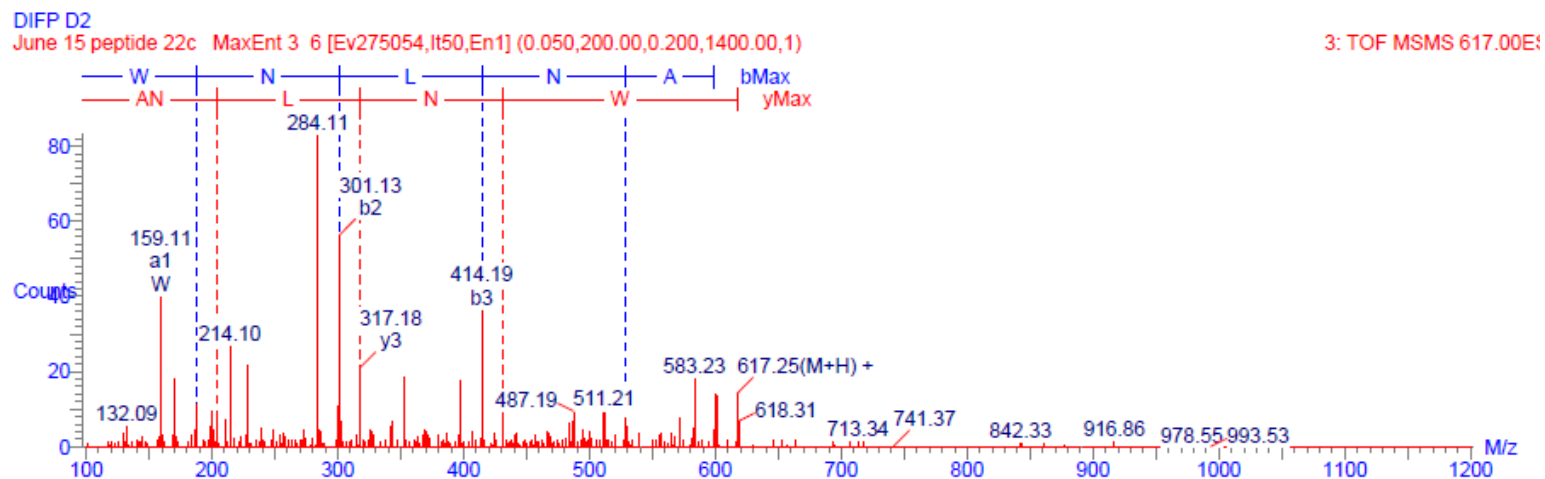


Figure A6. MS/MS spectra of the peptide Trp-Asn-Leu-Asn-Ala

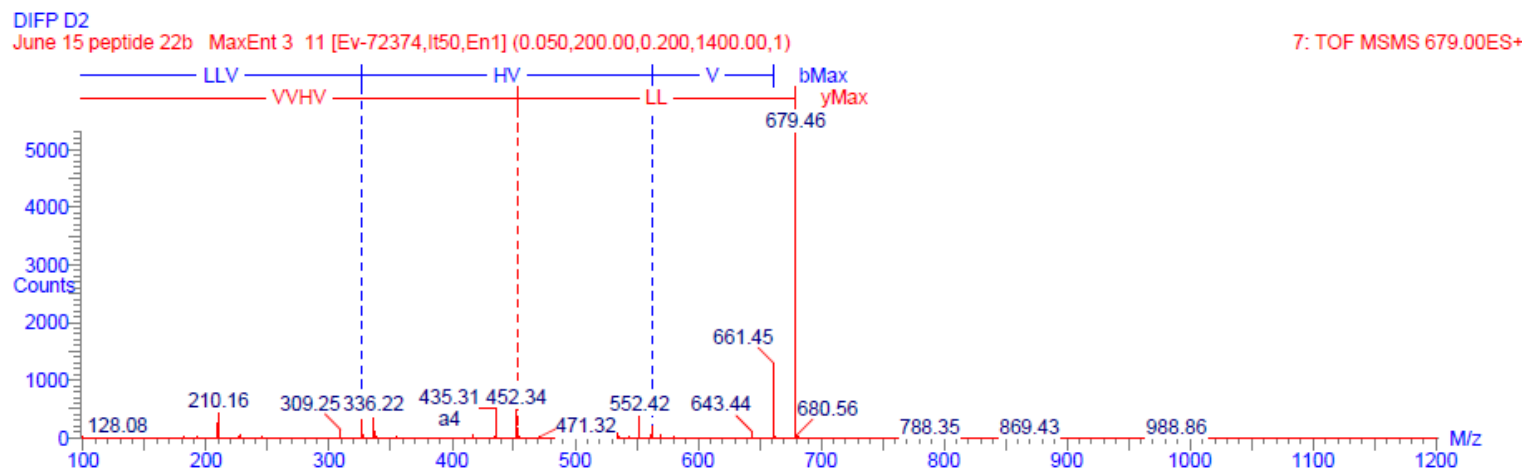


Figure A7. MS/MS spectra of the peptide Leu-Leu-Val-His-Val-Val

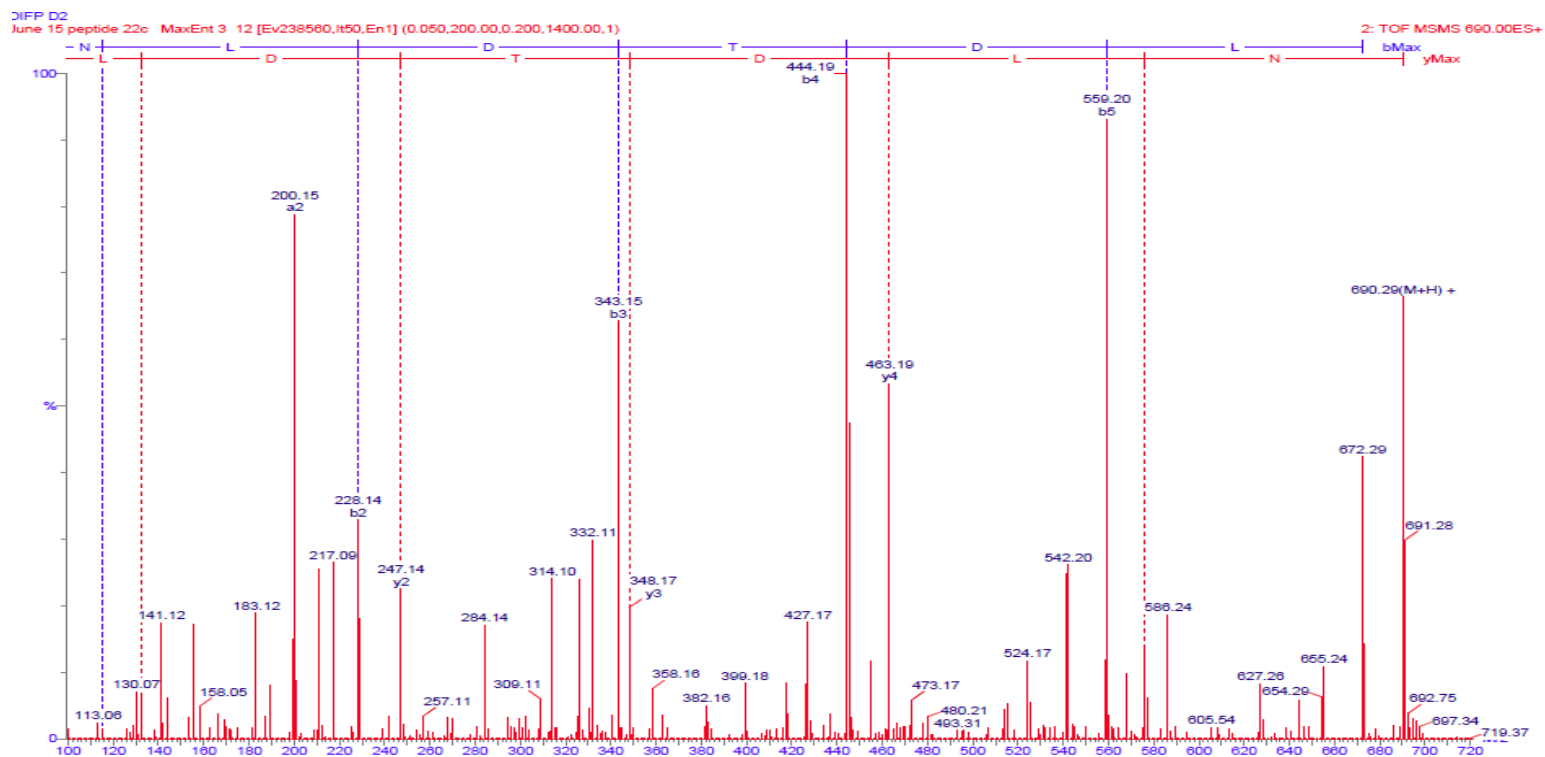


Figure A8. MS/MS spectra of the peptide Asn-Leu-Asp-Thr-Asp-Leu

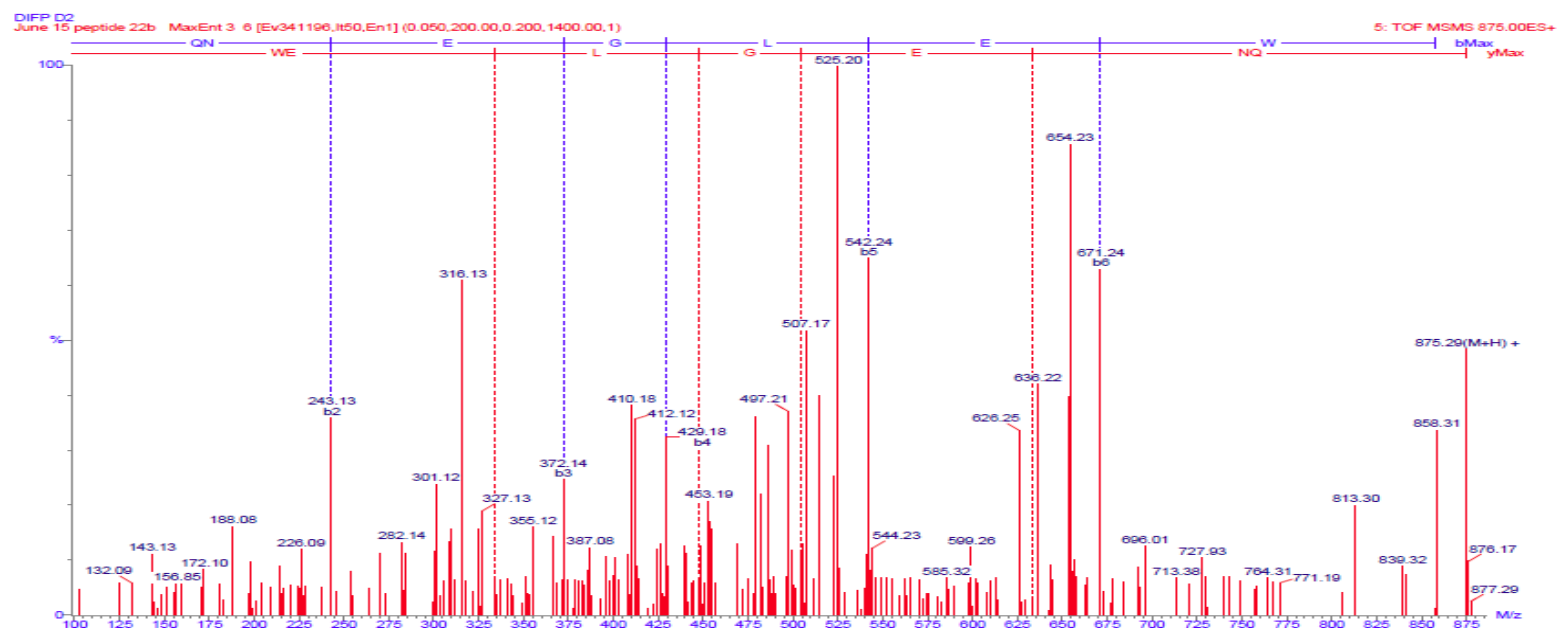


Figure A9. MS/MS spectra of the peptide Gln-Asn-Glu-Gly-Leu-Glu-Trp

MS/MS spectra of the most abundant peptides identified in the fraction of FPH (DH: 11.94 %, molecular mass range: 0.5-1.05 kDa) with the highest ACEI activity.

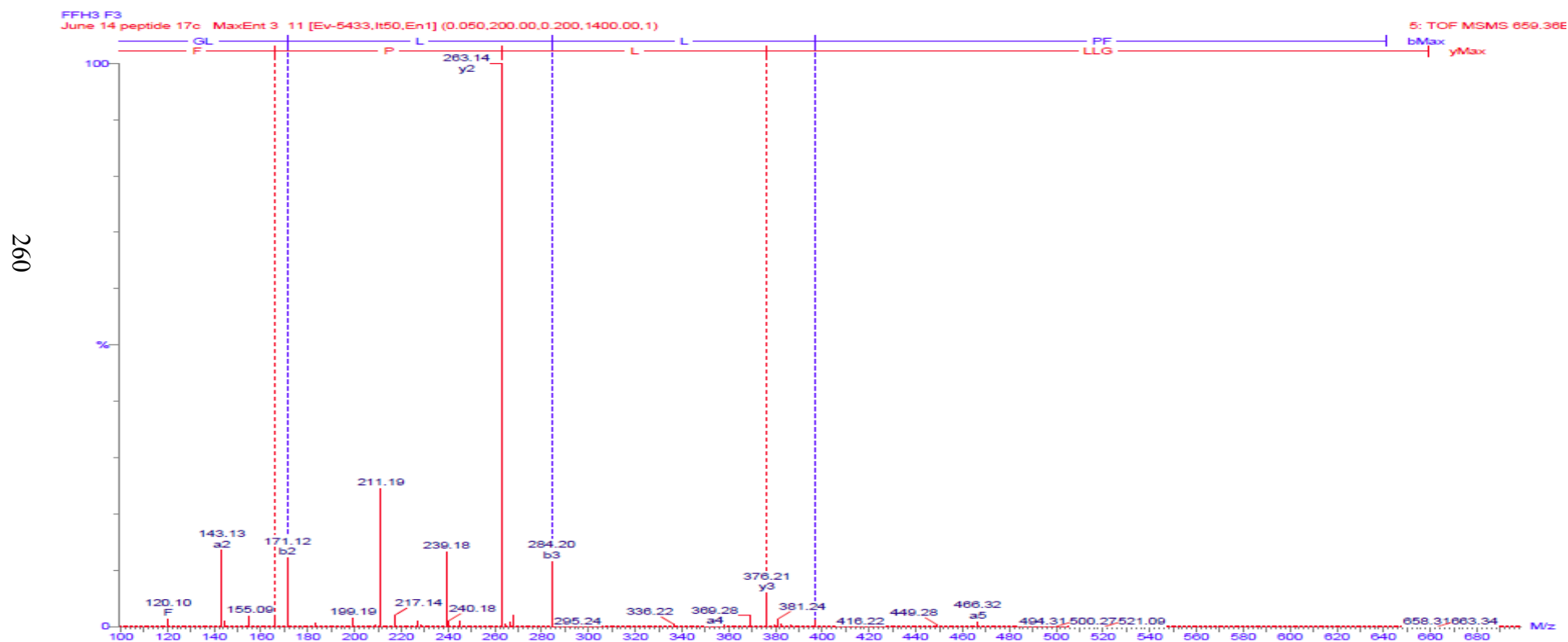


Figure A10. MS/MS spectra of the peptide Gly-Leu-Leu-Leu-Pro-Phe

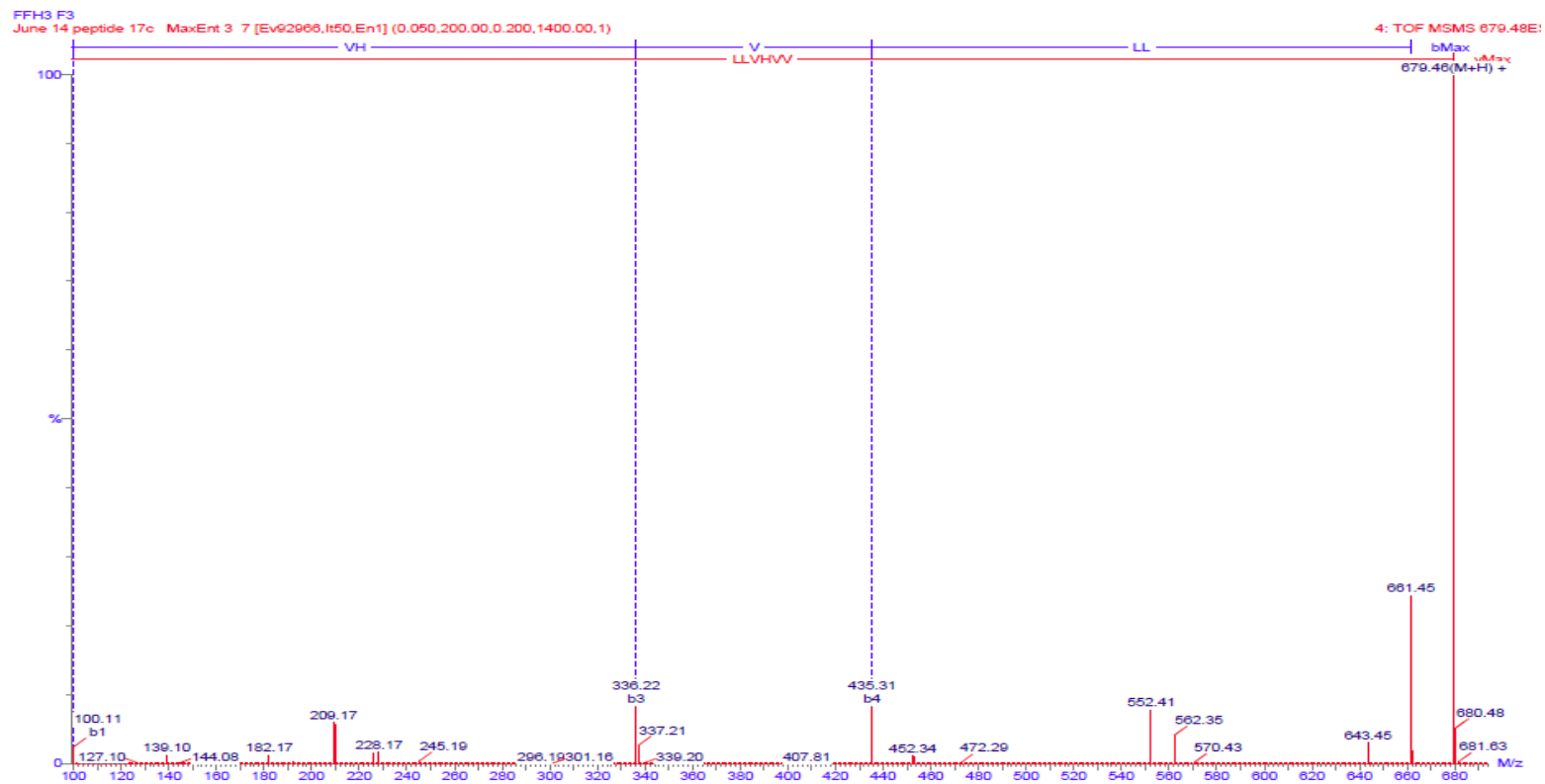


Figure A11. MS/MS spectra of the peptide Val-Val-His-Val-Leu-Leu

MS/MS spectra of the most abundant peptides identified in the fraction of FPH (DH: 24.63 %, molecular mass range: 0.5-1.05 kDa) with the highest OH[•] scavenging activity.

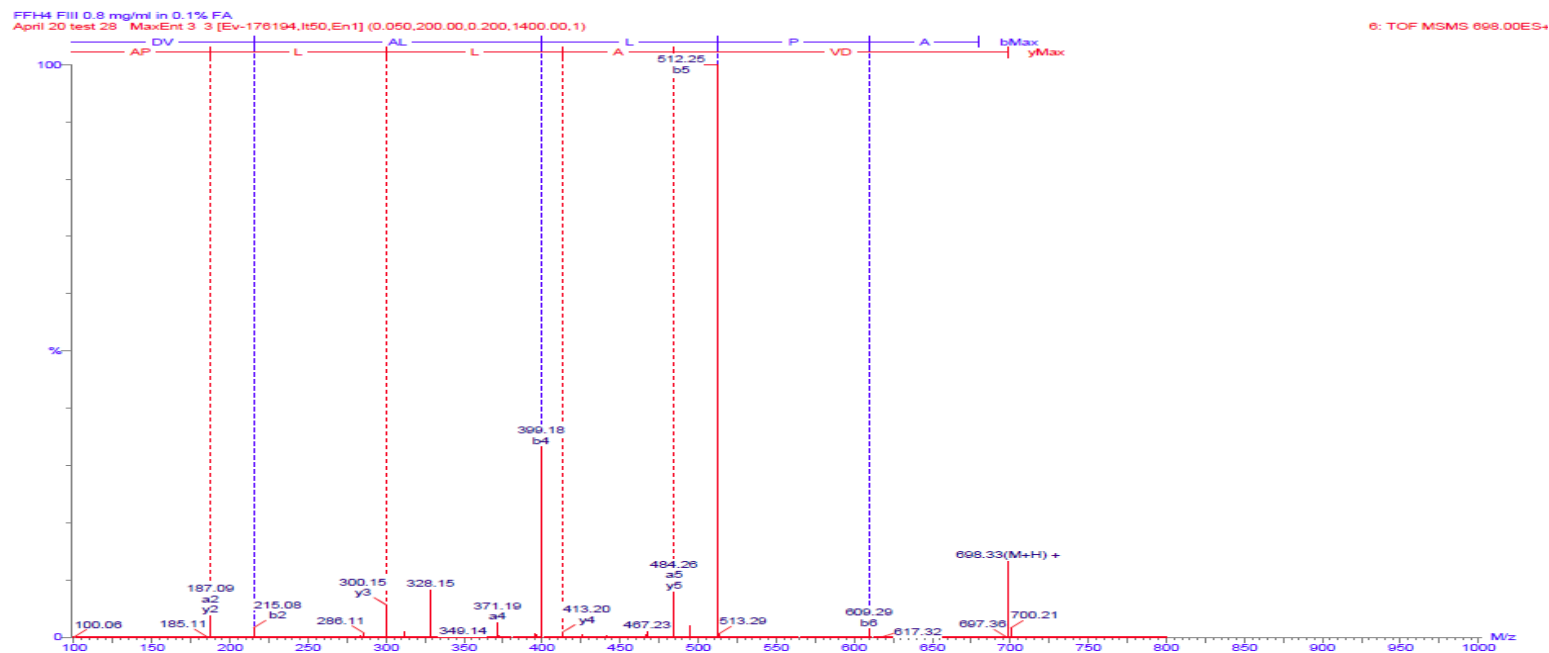


Figure A12. MS/MS spectra of the peptide Asp-Val-Ala-Leu-Leu-Pro-Ala

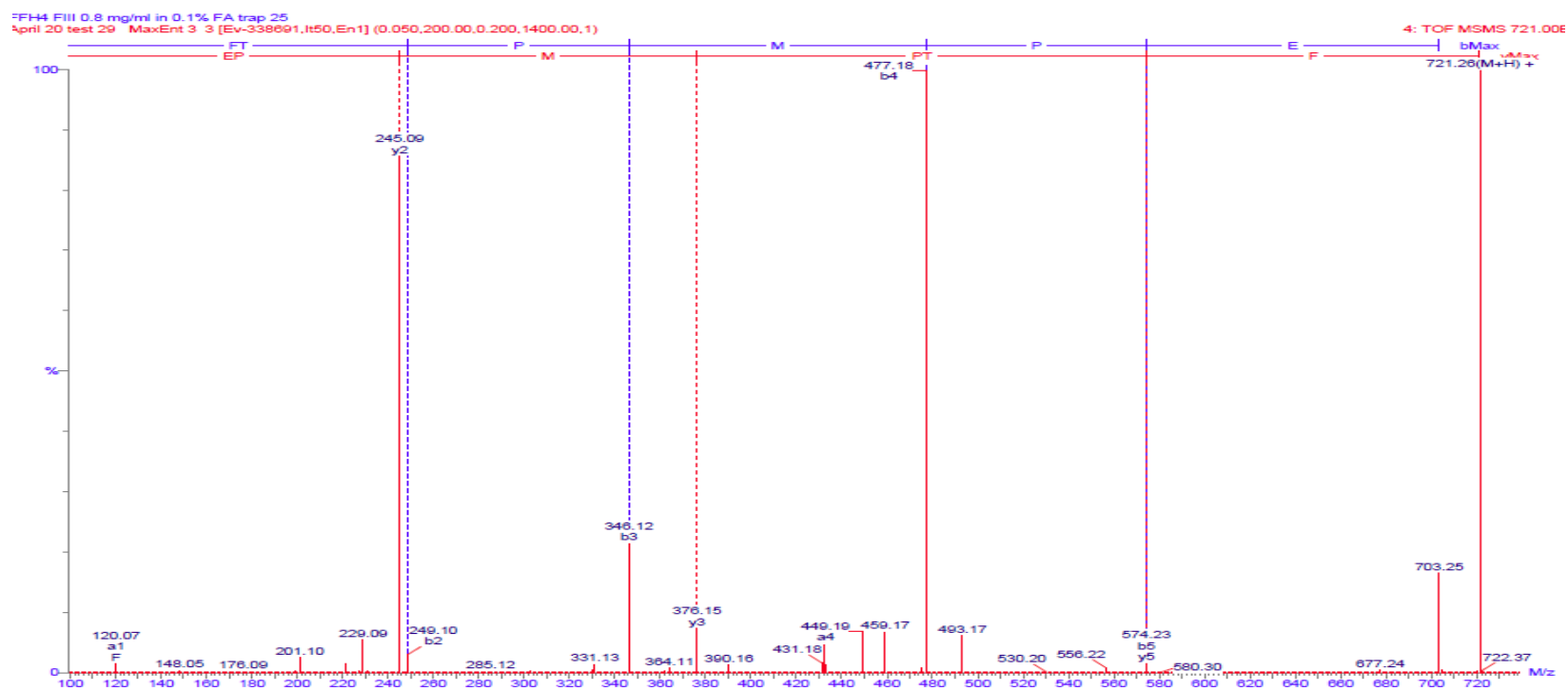


Figure A13. MS/MS spectra of the peptide Phe-Thr-pro-Met-Pro-Glu

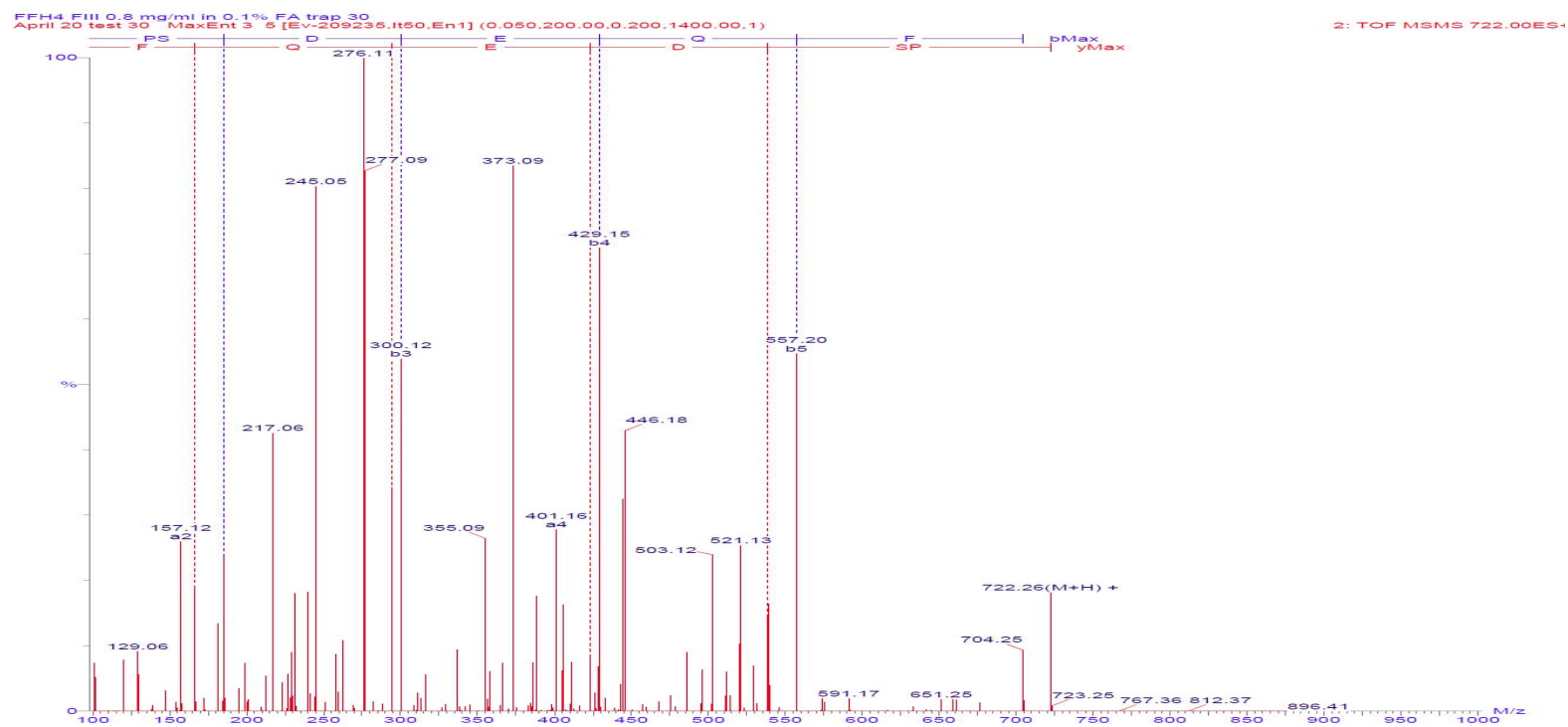


Figure A14. MS/MS spectra of the peptide Pro-Ser-Asp-Glu-Gln-Phe

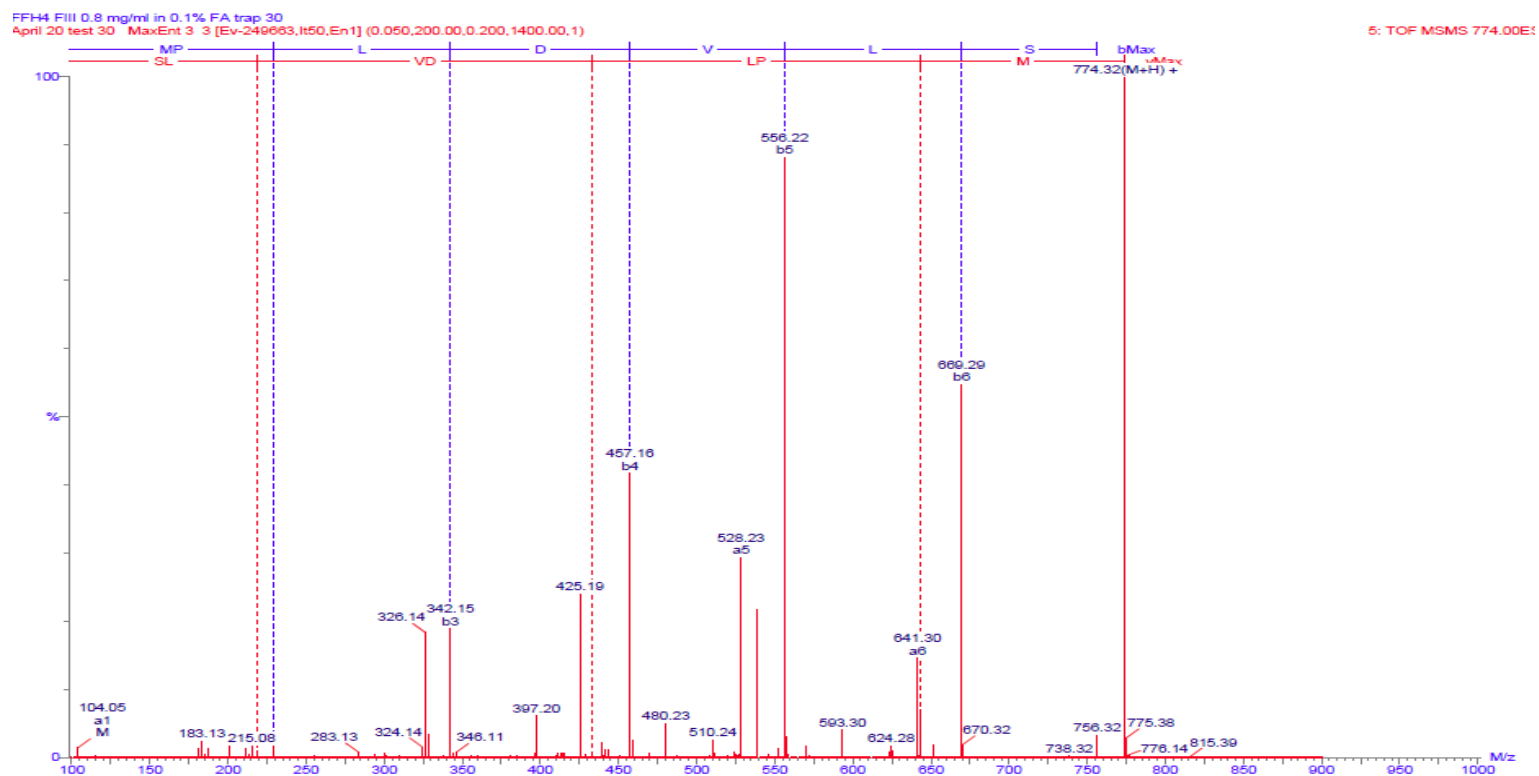


Figure A15. MS/MS spectra of the peptide Met-Pro-Leu-Asp-Val-Leu-Ser

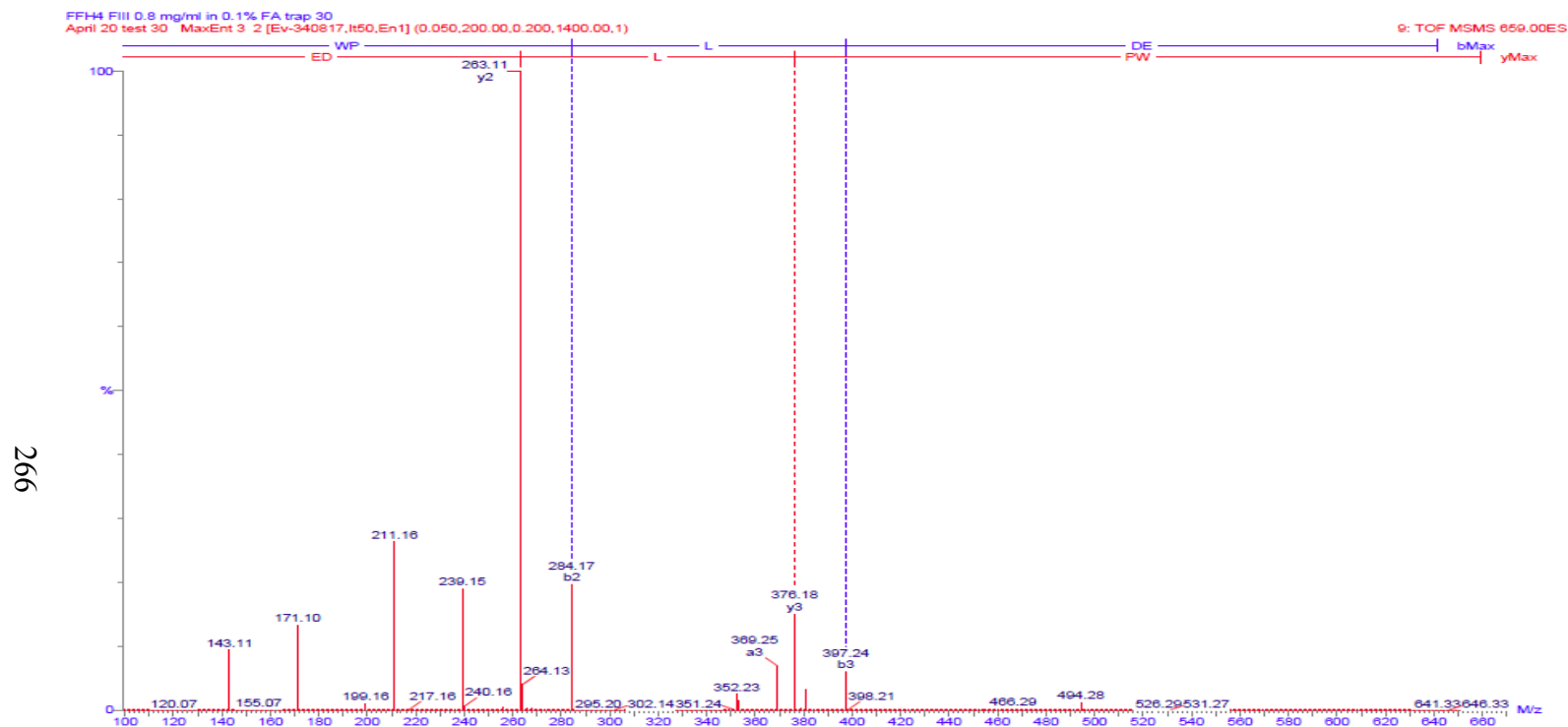


Figure A16. MS/MS spectra of the peptide Trp-Pro-Leu-Asp-Glu